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SELMAN A. WAKSMAN *was born July 2, 1888, in Priluka, a small town in the Ukraine, Russia. His parents were* JACOB *and* FRADIA (LONDON) WAKSMAN. *After graduating in 1910 from the Fifth Latin School in Odessa, he left for the United States.*

He entered the College of Agriculture of Rutgers University in 1911 and received his bachelor of science degree in 1915. He became a naturalized citizen the same year. He then was appointed research assistant in soil microbiology at the New Jersey Agricultural Experiment Station, and later Research Fellow at the University of California. He obtained a master of science degree from Rutgers University in 1916 and a doctor of philosophy degree from the University of California in 1918.

The same year, Dr. WAKSMAN *received an appointment as Microbiologist at the New Jersey Agricultural Experiment Station at New Brunswick, New Jersey, and as lecturer in soil microbiology at Rutgers University. He became associate professor in 1925, and in 1930 was made professor. He now is the head of the Microbiology Department of the College of Agriculture and Experiment Station at Rutgers University.*

In 1931, he was invited to organize a division of marine bacteriology at the newly established Woods Hole Oceanographic Institution and was appointed marine bacteriologist of that institution.

He is a member, honorary member, or fellow of a number of scientific societies in this country and abroad (Brazil, France, Germany, India, Mexico, Russia, Sweden). Among the American societies to which he belongs are the Society of American Bacteriologists, of which he is a former president, the National Academy of Sciences, and the National Research Council. He won the Nitrate of Soda Nitrogen Research Award in 1929, was president of Commission III on Soil Microbiology of the International Society of Soil Science (1927-1935), and was elected a corresponding member of the French Academy of Sciences in 1937.

In the summer of 1946, Dr. WAKSMAN *lectured before scientific groups in Europe and was given an honorary degree of doctor of medicine by the University of Liège in Belgium. He holds also honorary degrees of doctor of science, awarded to him by Rutgers in 1942 and by Princeton University in 1947, and an honorary degree of doctor of laws from Yeshiva University, New York, in 1948.*

Dr. WAKSMAN*'s work in his field has been recognized by several scientific societies in recent years. He received the Passano Foundation Award in 1947; the Emil Christian Hansen medal and award from the Carlsberg Laboratories in Denmark the same year; the New Jersey Agricultural Society medal; the Albert and Mary Lasker Award by the American Public Health Association, and the Amory Award by the American Academy of Sciences, all in 1948.*

He has published more than 300 scientific papers, and has written, alone or with others, eight books. Among these are Enzymes *(1926),* Principles of Soil Microbiology *(1927, 1932),* The Soil and the Microbe *(1932),* Humus *(1936, 1938),* Microbial Antagonisms and Antibiotic Substances *(1945, 1947), and* The Literature on Streptomycin, 1944-1948 *(1948). Another recent work, edited by Dr.* WAKSMAN, *is* Streptomycin—Nature and Practical Applications.

ANNALES CRYPTOGAMICI et PHYTOPATHOLOGICI

Volume 9

THE ACTINOMYCETES

ANNALES CRYPTOGAMICI

et PHYTOPATHOLOGICI

(incorporating Annales Bryologici)

edited by

FRANS VERDOORN, PH.D.

Managing Editor, Chronica Botanica
Research Fellow, Arnold Arboretum, Harvard University
Botanical Secretary, International Union of Biological Sciences

Wij en konnen den Heer en maker van het geheel Al niet meer verheerlijken, als dat wij in alle zaken, hoe klein die ook in onse bloote oogen mogen zijn, als ze maar leven en wasdom hebben ontfangen, zijn al wijsheit en volmaaktheit, met de uiterste verwondering sien uit steken.

Antoni van Leeuwenhoek

1950

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THE ACTINOMYCETES

Their Nature, Occurrence, Activities, and Importance

by

SELMAN A. WAKSMAN, PH.D.

Professor of Microbiology, Rutgers University
Microbiologist, New Jersey Agricultural Experiment Station

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PREFACE

Three and a half decades ago—in the spring of 1914—the writer, then a senior at Rutgers College, dug a spade into the earth of the New Jersey Agricultural Station experimental plots, to study the distribution of different groups of microorganisms occurring at different depths in the soil. This operation was repeated monthly, and sterile soil samples were removed to the laboratory and examined by use of ordinary plating procedures. A relatively simple agar medium was used.

Among the soil organisms that attracted the particular attention of the youthful investigator were the actinomycetes. Although he also enumerated the bacteria and the filamentous fungi, he was struck primarily by this much-neglected group of soil inhabitants, frequently spoken of as ray fungi and said to belong to the genus Actinomyces *or* Streptothrix. *On December 28, 1915, he presented before the 17th Annual Meeting of the Society of American Bacteriologists, a paper on the subject of "Bacteria, actinomyces and fungi in the soil." In this, his first contribution to the knowledge of the microbiological population of the soil, he said:*

"The actinomyces grow very slowly; they begin to develop from the bottom of the plate, and to the casual observer the colonies formed look like those of bacteria, even after 5-6 days' incubation; only the somewhat mealy or rough surface will disclose the fact that they are not bacteria. It requires careful observation to tell whether those white, pink or grey colonies are bacteria or not. Many counts of bacteria might have been confused, when this point was not known, and the fungi and actinomycetes were not taken into consideration."

Since this early survey of the occurrence and abundance of actinomycetes at different soil depths and in different soil types, the writer and his numerous associates and students have devoted much time to the study of the actinomycetes, their cultural characteristics, recognition of type species, their classification, their physiological properties and biochemical activities, their importance in the decomposition of pure organic compounds as well as of complex plant and animal residues in soils, peats, and composts, and finally their ability to produce antibiotic substances.

The writer has thus been concerned, during virtually his entire scientific lifetime, with the study of the actinomycetes. In summarizing our present knowledge of this interesting and important group of microorganisms, he has attempted to assemble the work of other investigators, with somewhat greater emphasis upon the work done in the laboratories

of the Department of Microbiology of Rutgers University and the New Jersey Agricultural Experiment Station.

To his many associates, who have helped in making this work possible, the author wishes to express his sincere appreciation for their unfailing enthusiasm and continuous interest and collaboration.

The writer also wishes to express his gratitude to Lt. Col. M. L. Littman *of the Armed Forces Institute of Pathology, for supplying various photographs, to Dr.* E. W. Emmons *of the National Institute of Health, for reading Chapter XI, and to Dr.* R. W. Goss *of the University of Nebraska for reading Chapter X.*

December 20, 1949 New Brunswick, N. J.

THE COMPOST

O how can it be that the ground does not sicken?
How can you be alive, you growths of spring?
How can you furnish health, you blood of herbs, roots, orchards, grain?
Are they not continually putting distemper'd corpses within you?
Is not every continent work'd over and over with sour dead?
Where have you disposed of their carcasses?
I do not see any of it upon you today—or perhaps I am deceiv'd.

Behold this compost! behold it well!
Perhaps every mite has once form'd part of a sick person—Yet behold!
The grass of spring covers the prairies,
The summer growth is innocent and disdainful above all those strata of sour dead.
What chemistry!
That the winds are really not infectious,
That when I recline on the grass I do not catch any disease,
Though probably every spear of grass rises out of what was once a catching disease.

Now I am terrified at the Earth! it is that calm and patient,
It turns harmless and stainless on its axis, with such endless successions of diseas'd corpses,
It distils such exquisite winds out of such infused fetor,
It gives such divine materials to men, and accepts such leavings from them at last.

WALT WHITMAN.

CONTENTS

Chapter XII

Appendix

Bacteria belong to the most wide-spread of organisms; we may say they are omnipresent; they never fail either in air or water; they attach themselves to the surface of all firm bodies, but develop in masses only where decomposition, corruption, fermentation or putrefaction are present. (Ferdinand Cohn, transl. by C. S. Dolley).

LIST of ILLUSTRATIONS

Mycology is the Cinderella of Botany and has suffered the disadvantages of step-sisterhood. The rest of the family at one time or another has received recognition, and occasionally with little warrant except that of importunity. But Cinderella is now fully attired for the Ball. Indeed the carriage is waiting. She has all the characteristics which usually attract in that she has developed in a comely manner and has charms of which her devotees are aware, and—she can bring her quiver full of rations for the general good. May those who have served her faithfully benefit for their devotion. . . . (J. RAMSBOTTOM).

LIST of TABLES

Les S. chromogena *et* alba *sont des microbes très répandus dans la terre, surtout abondants dans les racines végétales et à leur surface. Je les trouvai dans le terreau de jardin jusqu'à 1 m. de profondeur; plus bas encore le nombre absolu de ces organismes n'est guère considérable, mais dépasse néanmoins celui des autres microbes du sol. Cela démontre leur résistance à l'égard des conditions défavorables pour leur nutrition.* (M. W. BEIJERINCK).

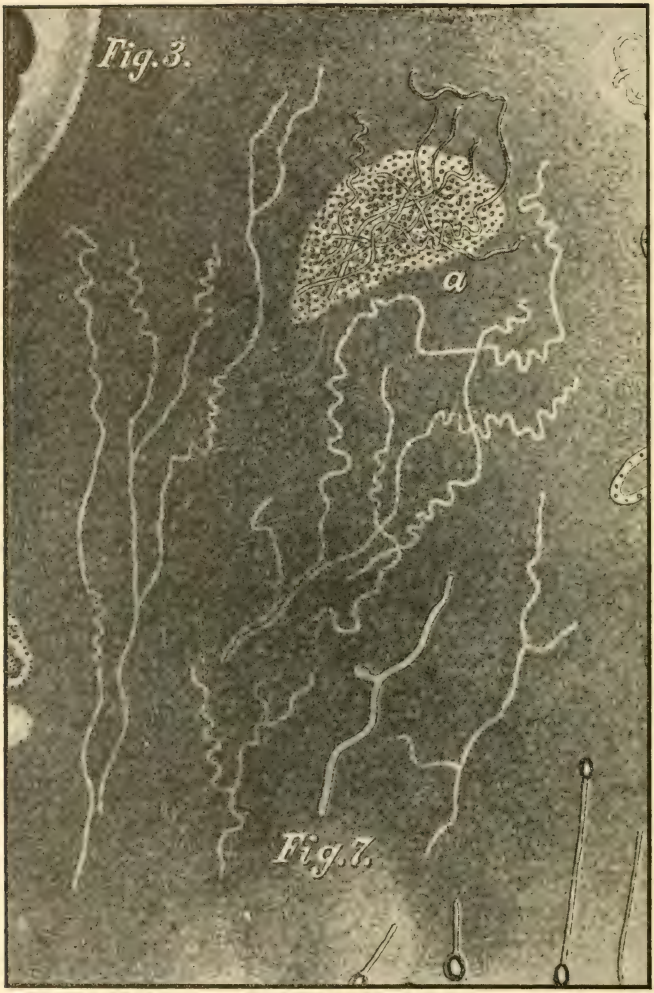

Fig. 1.—*Streptothrix* of Ferdinand Cohn. The first figures ever to have been published (1875) of an actinomyces (72).

INTRODUCTORY

Actinomycetes[1] comprise a group of branching unicellular organisms, which reproduce either by fission or by means of special conidia. They usually form a mycelium which may be of one kind—vegetative or substrate—or of two kinds—vegetative and aerial. The actinomycetes are related, on the one hand, to the true fungi or the *Hyphomycetes,* with which they have often been classified, and, on the other hand, to the true bacteria or the *Schizomycetes,* with which they are usually included for purposes of characterization and identification. In one of the early definitions of the actinomycetes (321) they were described as "unicellular microorganisms, 1μ in diameter, filamentous, branching monopodially, seldom dichotomous, producing colonies of radiating structure. They reproduce by fragmentation or oidia-formation; both kinds of spores grow in ordinary media to form filamentous mycelium, never growing into a rod-shaped vegetative state."

Frequently, the actinomycetes have been looked upon as a separate group of organisms occupying a position between the filamentous fungi and the bacteria. It has even been suggested that the actinomycetes be considered not only as forming the link between fungi and bacteria, but as representing the original prototypes from which both of these groups of organisms have been derived. Some of the actinomycetes are known to have their counterparts among the bacteria, and others among the fungi. The fact, however, that actinomyces mycelium and spores are similar in diameter to those of bacteria suggests the advisability of classifying the actinomycetes among the bacteria. A separate order has, therefore, been created, the *Actinomycetales,* which is distinct from the *Eubacteriales,* or the true bacteria.

Actinomycetes are of universal occurrence in nature. They are found in large numbers in soils, in fresh waters, in lake and river bottoms, in dust, on plant residues, on food products, in manures, and in composts. They are known to cause various important plant and animal diseases. Occasionally, they induce certain forms of food spoilage, especially because of the peculiar musty odor that they impart.

Notwithstanding an extensive literature dealing with the actinomycetes, many aspects of their nature and physiology, and even of their role in various natural processes, are still little understood. This is due to

[1] The word "actinomycetes" is used in this treatise to designate the organisms under discussion in a plural sense; the words "actinomyces" and "actinomycete" are used in a singular sense, without reference to any specific form, whether it be a member of the genus *Actinomyces,* or that of any of the other three genera.

certain factors, not the least among which is the confusion regarding their morphology, life cycles, and systematic position; the frequently assumed, although totally unjustified, difficulty of their cultivation and identification; and the meagre knowledge of their biochemical activities.

Numerous investigators have contributed much valuable information as to the nature and activities of the actinomycetes. This makes possible the recognition of a definite system for characterizing and for classifying these organisms. Information has also been accumulated concerning their physiology and their importance in natural processes. One particular property of these organisms, namely, their ability to produce a variety of antibiotic substances, has been utilized for a comprehensive series of investigations in numerous institutional and industrial laboratories. This has resulted in the isolation of certain agents, which have found application in combating a variety of bacterial infections in man and in animals.

Gradually it thus came to be recognized that the actinomycetes are a large and heterogeneous group of microorganisms, comprising several genera and many species. These organisms vary greatly in their physiology and in their role in natural processes. Together with the bacteria and fungi, they contribute to the cycle of life in nature, which results in the liberation, from the complex plant and animal residues, of a continuous stream of available elements, notably carbon and nitrogen, essential for fresh plant growth.

Chapter 1

TERMINOLOGY, PHYLOGENY, AND TAXONOMY

Because of their systematic position and their relationships to the bacteria, on the one hand, and to the fungi, on the other, much confusion has arisen concerning the taxonomic position of the actinomycetes This has been further complicated by the varied terminology used in different countries, and frequently even in the same country, to designate the genera and the species of this group of organisms.

The confusion is due to a number of factors, the most important of which may be summarized briefly as follows:

1. In 1875, Ferdinand Cohn (72) designated a culture of a filamentous organism found by R. Foerster in the concretions of the lacrymal duct as *Streptothrix Foersteri*. Cohn emphasized the similarity of this organism to the false-branching *Leptothrix*, on the one hand, and to the true-branching fungi on the other. The photograph of the organism as prepared by Cohn (Fig. 1) leaves no doubt that this was a true actinomyces. Soon afterward, in 1877, an infectious agent in cattle discovered by Bollinger (42) was named by Harz (166) *Actinomyces bovis,* because the masses of filaments were arranged radially, which suggested the name "actinomyces" or "ray fungus." Neither of these two generic designations has been universally accepted, largely because the first name (*Streptothrix*) had been preempted, and the second (*Actinomyces*) has been meeting with much criticism, because the description of the organism was based on its etiology rather than its morphology and cultural characteristics, and furthermore no pure culture was obtained.

2. Following these two basic contributions to our knowledge of the actinomycetes, numerous investigators, comprising medical workers, plant pathologists, botanists, and bacteriologists, devoted themselves to the study of this group of organisms. This resulted in various overlapping descriptions which frequently proved highly confusing, since different workers were interested in different aspects of the morphology, physiology, or etiology of the organisms concerned.

3. A large number of generic names were soon added to the first two, without sufficient consideration being given to the fundamental aspects of the morphology and physiology of the organisms themselves. The increasing number of generic designations were then further complicated by a large number of species descriptions. These were based either upon the natural substrate from which the organisms were isolated or upon a single physiological property, such as odor or pigment production when grown in a complex organic medium.

4. It has now been established that we are dealing here, not with a few species of a highly specialized and limited group of organisms, but with a large and heterogeneous group comprising many thousands of species which occur in numerous natural substrates and which take part in many natural processes. Because of this, it has been generally felt that a more comprehensive study of these organisms and the separation of the group into several genera were justified. One of the main difficulties, however, was the problem of digesting a most extensive literature.

Until very recently, too little was known of the morphology and physiology of the actinomycetes to justify recognition of basic differences between the different forms in an attempt to establish specific types. Most of the descriptions of the individual species were based largely upon cultural characteristics, usually growth on media highly complex in composition. Production of pigment in the mycelium of the organism and excretion of the pigment into the medium were considered among the most important distinctive characters. The presence or absence of growth on certain media, the liquefaction of gelatin, the digestion of milk proteins, and the production of odor were regarded as other distinguishing features. Insufficient recognition was given to the fact that these characteristics varied greatly under different conditions of cultivation, such as composition of the medium, oxygen supply, and temperature of incubation of the culture. The fact that an organism may undergo various cultural changes when grown for some time on artificial media was also disregarded. Certain aspects of the life cycle of a culture, such as the phenomenon of lysis and the problems of variation and mutation, so common among these organisms, were not recognized at all.

In the face of these shortcomings, the difficulty of establishing type cultures is understandable. In most cases, it was much easier to designate any freshly isolated strain by a new name than to identify it with a previously established species. Since the comparisons were usually made not with type cultures but with written descriptions, which were frequently quite inadequate, the resulting confusion is not surprising. Through the years, many new names accumulated, with the resulting difficulty of recognizing the relations of the designated organisms to older or previously described types.

With all these limitations, however, information was gradually accumulating concerning proper methods of growing actinomycetes on synthetic media. The specific morphology of different forms was becoming established. This helped in recognizing the true systematic position of the group, and pointed to its separation into several distinct and easily recognizable types, which could be raised to the status of genera.

No attempt will be made to review in detail the early literature on the actinomycetes. Such reviews may be found in the monographs of Lieske (260), Orskov (328), Duché (98), Kriss (242), and Kras-

SILNIKOV (234, 236), and in many of the earlier (310) and more recent papers (17-20, 106-108, 111-113, 185-192). It is sufficient here to summarize briefly some of the outstanding facts which led to our present knowledge of the terminology and classification of the actinomycetes. More detailed information concerning the nature, occurrence, and importance of certain special groups, notably those that produce animal and plant diseases, the antagonistic forms, and the thermophilic types, will be found in other sections of this monograph.

A word must be said here concerning recognition of individual species. At one time it was believed that only very few species of actinomycetes are found in nature. This belief was based upon observations of the growth of these organisms on complex organic media or upon the appearance presented by the organisms in the substrate from which they were isolated. The presence of a white aerial mycelium was believed to indicate an *albus* type; production of a black or brown pigment led to recognition of the *chromogenus* type; production of a characteristic musty odor gave rise to the *odorifer* type; when a culture was isolated from an actinomycotic lesion, it was called the *bovis* type, while an isolate from a scabby potato was considered as the *scabies* type. The introduction of differential, especially synthetic, media brought out the great variability of the actinomycetes. This frequently led to a multiplicity of names and descriptions based upon minor cultural differences on various media. Thus, we have names after all the colors of the rainbow, such as "albus," "ruber," "roseus," "flavus," "glaucus," "viridis," "lavendulae," "violaceus," "cyaneus," "niger," and many synonyms of these.

Fortunately, sufficient information has now accumulated on the morphology of the actinomycetes to justify the separation of this large and highly heterogeneous group of organisms into several genera; cultural, physiological, and often ecological characteristics may be utilized for their separation into species.

Synonyms of Generic Names of Actinomycetes:—It is hardly necessary to attempt a complete survey of all the generic and specific names that have ever been given to the group of actinomycetes as a whole or to certain constituent forms in particular. In some cases, these names have also been used to designate certain true fungi or true bacteria; in other cases, the names were simple synonyms. Some of the more common designations of the group and their historical significance are listed here:

1. *Actinomyces* Harz (1877).—The most widely used generic name for the actinomycetes is *Actinomyces*. It gave rise to the etiological designation of the disease actinomycosis, as well as to the name of the order as a whole, *Actinomycetales;* the common designation of this group of organisms, an *actinomyces* or an *actinomycete,* has also been derived from this name. It is made up of two Greek words, *actino,* meaning ray, and *myces,* meaning fungus. The specific name of the organism

was given as *Actinomyces bovis*. More detailed descriptions were presented in 1890 by Bostroem (44) and by Wolf and Israel (512), the latter having established that actinomycosis in man is caused by an anaerobic form, growing at 37°C., which is also infectious to animals. It has been suggested that this organism be divided into two forms, one causing human diseases, and the other, animal diseases. The reasons for and against the division will be presented later.

2. *Streptothrix* Cohn, F. (1875).—Although *Streptothrix* was the first name proposed for a true actinomyces, it has not received wide recognition. This is due largely to the fact that the name had been preempted: Corda used it in 1839 for a true fungus, which he designated *Streptothrix fusca*. Some of the early students of the actinomycetes (32) recognized this and insisted upon the greater justification of the designation *Actinomyces*. Another reason why the generic name *Streptothrix* has not been generally accepted is that Cohn himself failed to differentiate sufficiently between the organism to which he gave this name and the forms designated as *Cladothrix*, which are now known to be true bacteria.

3. *Cladothrix* Cohn, F.—The organisms recognized as *Cladothrix* Cohn represent a group of thread-forming, non-branching bacteria, which produce slimy capsules; they multiply by means of motile conidia (*Cladothrix dichotoma*) and are often found in mouths of animals. The use of this term by many of the early students of actinomycosis or pseudotuberculosis in man (109, 4) was soon disregarded.

4. *Leptothrix* Kützing, F. T. (1843).—The generic name *Leptothrix* has often been applied to the actinomycetes, although it was originally proposed and is now commonly used to designate a group of thread-forming, non-branching bacteria. These organisms embrace certain slime-forming iron-bacteria (*L. ochracea*) and various mouth-inhabiting bacteria (*L. buccalis*), which later came to be designated as *Leptotrichia* Trevisan (420).

5. *Discomyces* Rivolta (1878).—A certain amount of recognition has been accorded the generic name *Discomyces*. This name had previously been applied to a group of true fungi and has not, therefore, been generally accepted (98).

6. *Oospora* Wallroth, F. C. (1833).—The name *Oospora* also was first applied to a group of true fungi. Nevertheless, Sauvageau and Radais (384) attempted, in 1892, to describe the actinomycetes under this genus. Thaxter (415), as well, designated an important group of soil actinomycetes, namely, those that produce potato scab, by this generic name. It was later established that the causative agent of this disease belongs to the true actinomycetes (161).

7. *Nocardia* Trevisan (1889).—The generic name *Nocardia* was used to designate an organism belonging to the actinomycetes which was isolated by Nocard from "farcine du boeuf." Wright (519) proposed limitation of this name to a disease condition which is accompanied by

inflammation and which was, therefore, called nocardiosis, as distinct from actinomycosis. Pinoy (339) included the aerobic forms of actinomycetes under this generic name, a fact recognized in this treatise.

8. *Actinocladothrix* Afanassiev (1889).—This name was used to designate the causative agent of actinomycosis in man. Since it had no advantage over the name given by Harz, it has been but little used.

9. *Micromyces* Gruber, M. (1891).—The generic name *Micromyces* was applied to an organism (*M. hoffmanni*) which apparently belonged to the actinomycetes and which was isolated from the human body. This generic designation was not accepted by other investigators, since it represented no advantage over previous names, nor did it stand for a clearly recognized type (159).

10. *Actinobacterium* Haas, E. (1906).—To designate organisms that are intermediary between the true actinomycetes and the corynebacteria, the name *Actinobacterium* was suggested. It has not been generally accepted, although existence of these intermediate forms is not denied.

11. *Actinobacillus* Lingières and Spitz (1904) and *Actinobacillus* Brumpt (1910) were names applied to nocardia-like organisms, the true nature of which was not sufficiently recognized. The generic name was also used by Beijerinck, in 1914, for an organism which he had originally described in 1903 as *Bacillus oligocarbophilus* and for another called *Actinomyces* (*Streptothrix*) *paulotrophus*.

12. *Cohnistreptothrix* Pinoy, E. (1911).—In order to differentiate anaerobic actinomycetes from the aerobic forms, Pinoy used this name to designate the former. Castellani and Chalmers (66) as well as Langeron (250) accepted this designation; Orskov (328), however, applied the name to a group of aerobic actinomycetes.

13. *Anaeromyces* Castellani, A., Douglas, M. and Thompson, T. (1921).—This name was suggested to designate a group of organisms that are intermediary between the genera *Mycobacterium* and *Actinomyces*.

14. *Aerothrix* Wollenweber (1921) was used to designate those actinomycetes which produce aerial mycelium.

15. *Pionnothrix* Wollenweber (1921) was applied to those forms which do not produce aerial mycelium. This designation and the previous one have received no consideration because of a lack of sufficient characterization of the new genera, or rather subgenera, thus created. The production of aerial mycelium alone is not a sufficiently distinct characteristic to warrant separation of the actinomycetes into generic types, although it is a very important characteristic.

16. *Euactinomyces* Langeron, M. (1922).—This name was used to designate aerobic actinomycetes, as distinct from the anaerobic *Cohnistreptothrix*. It has no advantage over those previously suggested.

17. *Brevistreptothrix* Lignières (1924).—The generic name *Brevistreptothrix* was applied to the actinomycetes of the *A. hominis* group.

18. *Proactinomyces* Jensen (1931).—This name was used to desig-

nate a certain group of actinomycetes, characterized by a special manner of sporulation, as will be described later. These forms are somewhat related to the genera *Corynebacterium* and *Mycobacterium*. The organisms belonging to the genus *Proactinomyces* were later included by Lehmann and Haag in a separate family Proactinomycetaceae. This group includes such important forms as *A. hominis* Wolf-Israel, *Streptothrix israeli* Kruse, *A. farcinicus,* and *A. asteroides*. The name *Nocardia,* however, appears to deserve priority in designating this group of actinomycetes.

19. *Micromonospora* Orskov (1923).—The generic name *Micromonospora* was used to designate those actinomycetes that produce single spores on side branches. *Streptothrix chalcea* of Foulerton and *A. monosporus* of Lehmann and Schütze belong to this group.

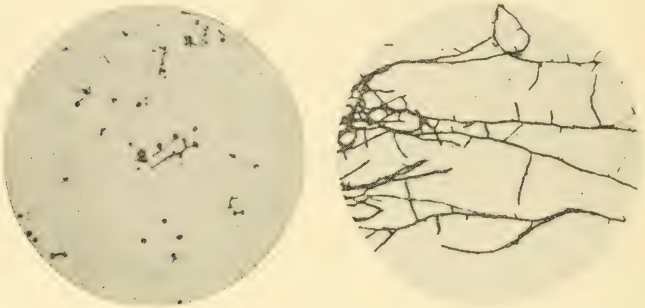

Fig. 2.—First photograph of a species of *Micromonospora* (1899). This organism was a thermophilic form growing in hot manure compost and called by Tsiklinsky *Thermoactinomyces vulgaris* (429).

20. *Thermoactinomyces* Tsiklinsky (1899).—This name was first used to designate thermophilic actinomycetes. Although one of the forms included in this group is definitely a *Micromonospora,* as shown in Fig. 2, the fact that forms producing the long-chain type were also included would preclude the use of *Thermoactinomyces* as the generic name. The separation of the thermophilic forms into a separate genus is hardly justified, since organisms which definitely belong to several different genera would be included. The temperature tolerance of certain types of actinomycetes is commonly used only for species separation and not for separation of genera.

21. *Mycococcus* Bokor (1930).—This name was first used to designate certain nocardia-like organisms (41). It was later applied by Krassilnikov (234) to certain bacteria which appear to be related to the actinomycetes.

22. *Asteroides* Puntoni and Leonardi (1935).—This name was also

used to designate certain members of the nocardia group of actinomycetes.

23. *Streptomyces* Waksman and Henrici (1943).—In order to separate those aerobic and nonpathogenic actinomycetes which produce aerial mycelium and which multiply by forming true conidia in chains, from the anaerobic forms, on the one hand, and from the nonconidial types and the single-spore types, on the other, this name was proposed. The generic designation combines the first two names given to the actinomycetes and which have been most commonly employed in micro-

Table 1: *Types of actinomycetes recognized in 1894* (132):—

Name	Observer	Synonym	Observer
A. bovis sulphureus	Rivolta	*A. bovis* (?)	–
A. foersteri	Cohn	*Streptothrix foersteri*	–
A. canis	Vachetta	*A. pleuriticus canis familiaris*	Rivolta
		A. canis	Rabe
A. bovis farcinicus	Nocard	*Bacillus farcinicus*	–
A. cati	Rivolta	–	–
A. bovis albus	Gasperini	*Streptothrix* 1,2,3	Almquist
		S. alba	Rossi-Doria
A. astercides	Eppinger	*Cladothrix astercides*	–
		S. astercides	Gasperini
		S. eppingerii	Rossi-Doria
A. chromogenus	Gasperini	*S. chromogenus*	–
		S. niger	Rossi-Doria
		Oospora metschnikowi (?)	Sauvageau and Radais
		O. guignardi (?)	Sauvageau and Radais
A. bovis luteo-roseus	Gasperini	–	–
A. cuniculi	Schmorl	*S. cuniculi*	–
A. hoffmanni	Gruber	*Micromyces hofmanni*	–
A. albido-flavus	Rossi-Doria	*S. albido-flava*	–
A. violaceus	Rossi-Doria	*S. violacea*	–
A. carneus	Rossi-Doria	*S. carnea*	–
A. citreus	Gasperini	–	–
A. pluricolor (?)	Terni	–	–
A. arborescens	Edington	–	–
A. ferrugineus	Naunyn	–	–

biological literature, *Streptothrix* and *Actinomyces*. This new name obviates the need for using the name *Streptothrix*, for reasons indicated above, and reserves the designation *Actinomyces* for the true anaerobic forms to which it was first applied.

In addition to the above designations, various other generic names have been used, at one time or another, to designate all the actinomycetes or certain constituent groups. These include *Actinococcus, Actinophyta, Bollingera, Indiella, Indiellopsis, Microsiphonales, Microsporum, Oidium,* and others. Either these names proved to be mere synonyms or they could not be given serious consideration for various other reasons.

As early as 1894, a number of species were already recognized. The names of many of them were considered as synonymous, as shown in Table 1.

Systematic Position and Classification of Actinomycetes:—

Relation of actinomycetes to bacteria and fungi.—Although they are very often grouped with the fungi, the actinomycetes are related in many respects to the bacteria and are usually classified with the bacteria under the Schizomycetes.

The relationship of the actinomycetes to the bacteria is based upon the following properties:

1. The diameters of the filaments and spores of actinomycetes are similar to those of true bacteria and not of fungi.
2. Many actinomycetes reproduce by fragments or oidia that are similar in size and in shape to the rod-shaped and spherical bacteria.
3. Many actinomycetes, especially the pathogens, produce no aerial mycelium; their growth appears similar to that of pleomorphic bacteria, like the members of the genus *Corynebacterium.*
4. Many actinomycetes are acid-fast, and in their morphology and physiology resemble true bacteria, namely, the members of the genus *Mycobacterium.* Certain groups among the actinomycetes, especially the genera *Actinomyces* and *Nocardia,* show a close resemblance to the mycobacteria.

That the actinomycetes show a definite relationship to the fungi, especially the Fungi Imperfecti, is brought out by the following properties:

1. The manner of branching of the aerial mycelium of many representative groups of actinomycetes, especially the genera *Streptomyces* and *Micromonospora,* definitely resembles that of fungi.
2. The production by a large number of actinomycetes of an aerial mycelium and of conidia is definitely typical of many true fungi.
3. The growth of the colonies on the surfaces of liquid and of solid media, as well as their growth in a suspended or submerged condition, is similar to that of fungi and not of true bacteria. Turbidity is not usually produced in the liquid culture.

One may, therefore, conclude that the actinomycetes comprise many highly heterogeneous groups of organisms, varying greatly in their morphological characteristics, and resembling in some respects true bacteria and in others true fungi. For these reasons, the actinomycetes may tentatively be placed in a taxonomical transition group between the *Schizomycetes* and *Hyphomycetes,* with considerable similarity to, if not actual overlapping of, one or the other.

Classification of actinomycetes.—Many systems of classifying the actinomycetes have been suggested. These are based upon their activities in a natural environment such as pathogenic and nonpathogenic forms, upon their cultural characteristics such as pigmentation and gela-

tin liquefaction, or upon their morphology, especially the manner of sporulation.

Buchanan (52) suggested placing the actinomycetes in the order *Actinomycetales* with a single family *Actinomycetaceae*. The latter was divided into four genera, *Actinobacillus, Leptotrichia, Actinomyces,* and *Nocardia*. Later (53), in the early editions of Bergey's Manual, the *Actinomycetales* were divided into two families, the *Actinomycetaceae* with the genera *Actinobacillus, Leptotrichia, Actinomyces,* and *Ery-*

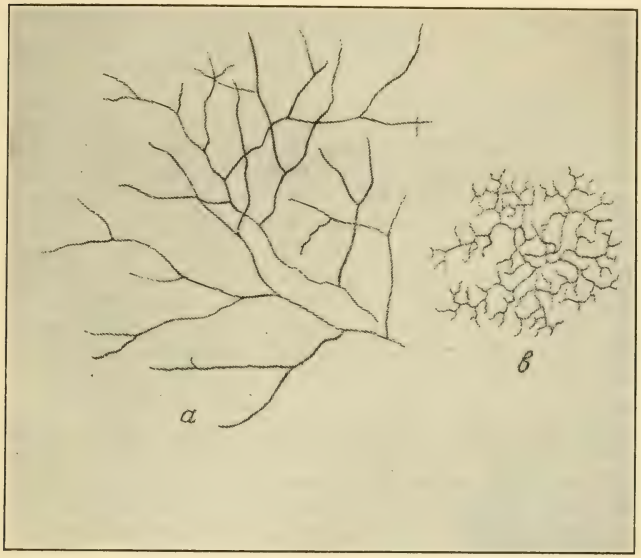

Fig. 3.—Structure of actinomyces mycelium: (*a*) *S. albus* Gasperini; (*b*) *S. aurantiacus* Gasperini (*from* Krassilnikov, 234).

sipelothrix, and the *Mycobacteriaceae* with eight genera, including *Mycobacterium*. In later editions of Bergey's Manual, *Actinobacillus* was dropped from the first family and *Proactinomyces* added; the second family was divided into the genera *Corynebacterium* and *Mycobacterium*. Further changes were made in the final, or sixth edition of the Manual.

Lehmann and Neumann (256) divided the order into two families, the *Proactinomycetaceae* with the genera *Corynebacterium* and *Mycobacterium,* and the *Actinomycetaceae* with a single genus *Actinomyces*. Kluyver and van Niel (224) suggested that the *Mycobacteriaceae* be removed altogether from the order *Actinomycetales*.

Several of the systems for classifying the true actinomycetes may be listed as follows:

A. *Classification of Schabad* (1904):

I. Non-acid-fast types, liquefying gelatin, producing granules in lesions, with typical swelling of hyphae. ..... *A. typica.*
II. Acid-fast types, not producing granules in lesions, without typical swellings of lesions. ..... *A. atypica.*
1. Gelatin liquefied. ..... *A. atypica simplex.*
a. ..... *A. alba.*
b. ..... *A. flava.*
2. Gelatin not liquefied. ..... *A. atypica pseudo-tuberculosa.*

B. *Classification of Krainsky* (1914):

1. Large colonies (3-5 mm.) produced on solid media; aerial mycelium typically pigmented; oval spores. ..... *Macroactinomyces.*
2. Small colonies (<3 mm.) produced on solid media; pigmented aerial mycelium; spherical spores. ..... *Microactinomyces.*
This system has been applied only to the saprophytic aerobic forms.

C. *Classification of Chalmers and Christopherson* (1916):

I. Granules black, noncultivable forms. .....
Actinomyces of Babes and Mironescu.
II. Granules white, yellow, orange, or red:
1. Cultivated with difficulty, anaerobic types, no arthrospores; granules in masses. ..... *Cohnistreptothrix.*
a. Granules yellow. ..... *C. israeli.*
b. Granules very small, white. ..... *C. thibiergi.*
2. Cultivated easily, aerobic types, arthrospores produced. ... *Nocardia.*
a. Clubs present. ..... *N. bovis.*
b. No clubs produced:
a^1. Granules surrounded by a hard shell. ..... *N. somaliensis.*
b^1. Granules without shell:
a^2. No growth on gelatin. ..... *N. krausei.*
b^2. Growth on gelatin:
a^3. Serum coagulated, liquefied:
a^4. Pathogenic to laboratory animals. .... *N. garteni.*
b^4. Nonpathogenic to laboratory animals:
a^5. Gelatin liquefied. ..... *N. liquefaciens.*
b^5. Gelatin not liquefied. ..... *N. convolutus.*
b^3. Serum not liquefied:
a^4. Culture yellow orange to red. ..... *N. asteroides.*
b^4. Culture white, then red. ..... *N. indica.*
This system was based entirely upon pathogenic forms.

D. *Classification of Waksman* I. (1919):

This system, like the previous one, was based largely upon the cultural characters of the organisms, embracing, however, mostly soil forms. Whereas the previous system (C) comprised the forms listed here under genera

Actinomyces and *Nocardia*, the cultures classified in this system (D) are now included under the genus *Streptomyces*.

E. *Classification of Wollenweber* (1921):

I. Weakly growing strains; aerial mycelium and conidia lacking. . . . . Subgenus *Pionnothrix*.
This group included *A. farcinicus, A. caprae, A. asteroides, A. polychromogenes*, and *A. pelletieri*.
II. More vigorously growing strains, producing aerial mycelium. . . . . Subgenus *Aerothrix*.
1. With sclerotial or spirodochial stroma. . . . . Section *Sclerostroma*.
This group included *A. bovis, A. foersteri, A. scabies*, and *A. aerugineus*.
2. Sclerotial or spirodochial stroma not significant.
a. With brown conidia. . . . . Section *Poliophaerospora*.
b. With light colored or colorless conidia. . . . . Section *Leucospora*.
a′. Substrate with variety of colors. . . . . Subsection *Heterochroma*.
a″. With spiral conidial chains. . . . . Series *Helicothrix*.
b″. Without spiral conidial chains. . . . . Series *Ahelicothrix*.
b′. Substrate with single color. . . . . Subsection *Monochromas*.
c. With reddish to red conidia. . . . . Section *Erythrinospora*.
d. With blue conidia. . . . . Section *Glaucospora*.

F. *Classification of Langeron* (1923):

I. Aerobic forms. . . . . *Euactinomyces*.
1. Forms parasitic to man and to animals. . . . . Section *Parasitica*.
a. Non-acid-fast, thin growth on solid media. . . . . *Majores*.
b. Acid-fast, tubercle-like growth on solid media. . . . . *Minores*.
c. Forms difficult to cultivate, not growing on potato, not liquefying gelatin or serum. . . . . *Breviores*.
II. Anaerobic forms. . . . . *Cohnistreptothrix*.
Facultative anaerobic forms, difficult to cultivate; no arthrospores produced.

G. *Classification of Orskov* (1923):

I. Typical conidia formation in aerial mycelium. . . . . *Cohnistreptothrix*.
II. Spore-formation by segmentation. . . . . *Actinomyces*.
III. Spores produced singly on branches of mycelium. . . . *Micromonospora*.

H. *Classification of Lignières* (1924):

I. Aerobic, long mycelium, not breaking up into rods. . . . . *Actinomyces*.
II. Anaerobic, short mycelium, breaking up into long rods. . . . . *Brevistreptothrix*.
III. No mycelium; cells rod-shaped. . . . . *Actinobacillus*.

I. *Classification of Jensen* (1931):

A. No spores formed. . . . . *Proactinomycetaceae*.
I. No mycelium formed:
1. Acid-fast organisms. . . . . *Mycobacterium*.

2. Non-acid-fast organisms. ..... *Corynebacterium.*
II. Mycelium formed. ..... *Proactinomyces.*
B. Spores formed. ..... *Actinomycetaceae.*
I. Spores in aerial mycelium. ..... *Actinomyces.*
II. Spores terminally on branches of vegetative mycelium. .....
Micromonospora.

J. *Classification of Duché* (1934):

This classification, like that of C and D, was based largely upon the cultural characteristics of the organisms, and like D, was limited to the conidia-producing aerobic types, especially of the *albus* group.

I. Vigorously growing forms.
1. Mycelium yellowish, no exopigment. Species included in this group, based on pigmentation of the mycelium, were *A. albus, A. alboviridis, A. roseus, A. halstedii, A. parvus, A. lavendulae.*
2. Mycelium yellowish, exopigment not very intense. Descriptions based on soluble pigment, such as *A. viridis, A. flavogriseus,* etc.
3. Mycelium black, no exopigment, white efflorescence. .. *A. reticuli.*
4. Mycelium yellow-red, no exopigment, white efflorescence .....
A. albosporeus.
5. Mycelium yellowish-clear, no exopigment, yellow efflorescence.....
A. citreus.

II. Non-vigorously growing forms:
1. Mycelium yellowish, no exopigment, poor yellowish efflorescence...
A. almquisti.

K. *Classification of Krassilnikov* (1938):

I. *Actinomycetaceae.*
1. Nonseptate mycelium, not breaking into rods. ..... *Actinomyces.*
2. Unicellular mycelium, later breaking into rods and cocci. .....
Proactinomyces.
3. No mycelium, elongated rod-shaped, branching and breaking into coccoid forms. ..... *Mycobacterium.*
4. Cells coccus-like, seldom rod-shaped; resting cells develop in a manner similar to actinomyces spores. ..... *Mycococcus.*

II. *Micromonosporaceae.*
Mycelium well developed; conidia produced singly on short conidiophores.
Micromonospora.

L. *Classification of Baldacci* (1939):

I. Filamentous, often producing two types of mycelium; no conidia formed; cells rod-shaped or coccoid; usually parasitic. ..... *Mycobacteriaceae.*
1. Rod-like organisms, rarely filamentous forms. .... *Leptotrichioideae.*
a. Thin, occasional mycelial hyphae, gram-negative.
a^1. Cells fusiform. ..... *Fusiformis.*
b^1. Cells rod-shaped or coccus-like. ..... *Actinobacillus.*
c^1. Cells rod-shaped, sometimes filamentous; branched. *Pfeifferella.*
b. Hyphae frequently present, gram-positive.
a^1. Filaments branched, thickened, showing characteristic granules.
Erysipelothrix.

b¹. Filaments unbranched, fragmented into short rods, sometimes with granules and septa. ..... *Leptotrichia.*
2. Filamentous, readily dividing into bacterial segments. ..... *Proactinomycoideae.*
a. Filaments with angular growth, dividing into bacteria-like segments.
a¹. Acid-fast. ..... *Mycobacterium.*
b¹. Not acid-fast. ..... *Corynebacterium.*
b. Long branching hyphae, filaments as in 2a; aerial mycelium may be present but not different from vegetative mycelium:
a¹. Anaerobic. ..... *Actinobacterium.*
b¹. Microaerobic, sometimes with sclerotial masses. *Cohnistreptothrix.*
c¹. Aerobic, well-developed mycelium, aerial and vegetative mycelium undifferentiated. ..... *Proactinomyces.*
II. Conidia always produced, with distinct aerial mycelium. ..... *Actinomycetaceae.*
1. Conidia produced singly. ..... *Micromonospora.*
2. Conidia seriated and multiple. ..... *Actinomyces.*

M. *Classification of Waksman* II. (1940):

I. Mycelium rudimentary or absent. ..... *Mycobacteriaceae.*
1. Nonmotile.
a. Acid-fast ..... *Mycobacterium.*
b. Non-acid-fast. ..... *Corynebacterium.*
2. Motile. ..... *Mycoplana.*
II. Mycelium produced.
1. Spores formed by segmentation. ..... *Proactinomycetaceae.*
a. Anaerobic forms. ..... *Cohnistreptothrix.*
b. Aerobes. ..... *Proactinomyces.*
III. Vegetative mycelium normally remaining undivided.
1. Conidia formed in chains from aerial hyphae. ... *Actinomycetaceae.*
Spores produced in chains. ..... *Actinomyces.*
2. Conidia formed terminally and singly on short conidiophores. ..... *Micromonosporaceae.*
Spores produced singly. ..... *Micromonospora.*

N. *Classification of Waksman and Henrici* (1943):

A. Mycelium rudimentary or absent. .....*Mycobacteriaceae* Chester.
I. Acid-fast organisms. ..... *Mycobacterium* Lehmann and Neumann.
B. True mycelium produced.
I. Vegetative mycelium fragmenting into bacillary or coccoid elements. *Actinomycetaceae* Buchanan.
a. Anaerobic or microaerophilic, parasitic, not acid-fast. ..... *Actinomyces* Harz.
b. Aerobic, partially acid-fast or non-acid-fast. ... *Nocardia* Trevisan.
II. Vegetative mycelium not fragmenting into bacillary or coccoid elements. ..... *Streptomycetaceae* Waksman and Henrici.
a. Multiplication by conidia in chains from aerial hyphae. ..... *Streptomyces* Waksman and Henrici.
b. Multiplication by single terminal spores on short sporophores. .... *Micromonospora* Orskov.

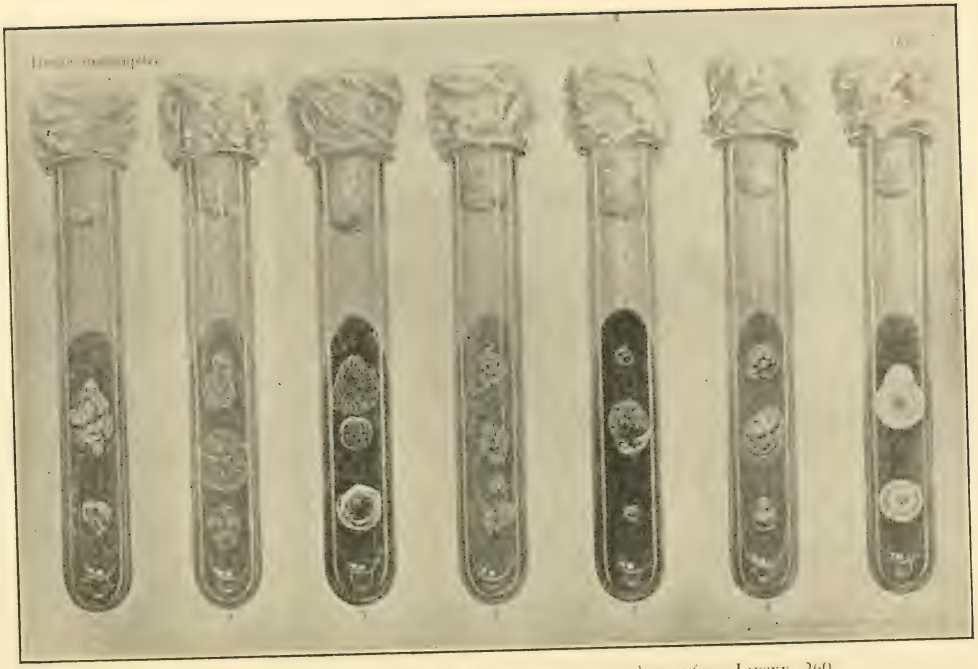

FIG. 4.—Typical growth of aerobic actinomycetes upon agar slants (*from* LIESKE, 260).

A detailed discussion of the various species that have so far been described among the actinomycetes, based upon the classification of the organisms into four genera is presented in the latest edition of Bergey's Manual (34). Additional species not included in this Manual are found in Krassilnikov's guide (236). The various principles upon which the recognition of individual species are based are outlined here. A description of the type species within each genus is also presented.

Identification of Actinomycetes:—To identify certain individual species of actinomycetes, it is sufficient to give recognition to some of their characteristic properties. These are based upon the occurrence of these organisms in their natural substrates, upon their morphology, upon their cultural characteristics, and upon their biochemical properties.

Ecology as a basis of classification.—Although various attempts have been made to classify actinomycetes into several groups on the basis of their natural habitats, no broad system for generalization can ever be developed on this basis alone. It is true that the anaerobic forms, described here under the genus *Actinomyces* are largely animal pathogens, and that some of the *Nocardia* species are also pathogenic. It is also true that the *Streptomyces* group is characteristic of soils and that the *Micromonospora* types are found in high-temperature composts, as well as in river and lake waters and bottoms. This alone is hardly sufficient for a separation of the organisms on the basis of their natural occurrence. Such a division would be arbitrary and only approximately true.

It has been suggested (250), for example, that the disease-producing actinomycetes be classified on the basis of the specific type of disease; namely, 1. anaerobes, of the Wolf-Israel type, that attack the abdomen; 2. aerobes that cause actinomycosis of the lungs, including saprophytes occurring in the dust; 3. forms causing swellings in the infectious area, organisms said to be of the so-called *Streptothrix* type.

Actinomycetes are universally present in water basins, in soils, in milk, in or upon other foodstuffs, and in dust. Many attempts have been made to divide these groups upon the basis of their specific habitats. The soil forms, for example, have been separated into plant pathogens and saprophytes; the food-inhabiting types, into odoriferous and non-odoriferous types. These separations, like those based on natural occurrence, were quite arbitrary. The cosmopolitan nature of many actinomycetes is well established, since species found in one part of the world, are soon discovered also in other parts. Species found in soils may also be found in peats or on foodstuffs. Thus ecology can hardly be considered as a major basis for the classification of actinomycetes.

Morphology as a basis for classification.—Although morphological characters are used for the separation of the broader groups of actinomycetes, the families and genera, they can also be utilized for the subdivi-

sion of these major groups into smaller units, the species. The nature of the aerial mycelium and the mode of spore formation are the two most distinguishing morphological characteristics. These were employed first by Drechsler, Orskov, and Waksman, and more recently by Jensen, Kriss, and Krassilnikov for the separation of species and even genera. These characters vary, however, and the limits of variation must be established.

Krassilnikov came to the conclusion, on the basis of comparative microscopic studies of many cultures freshly isolated, as well as cultures

Fig. 5 a-d.—Different types of branching of aerial mycelium of species of *Streptomyces:* above, long open spirals; Fig. 5b (p. 19), tuft formation of sporulating hyphae; Fig. 5c (p. 20), short compact spirals; Fig. 5d (p. 21), broom shaped structure of sporulating hyphae.

grown for 5 years on artificial media, that the form of the sporophores and of the spores is constant for every actinomyces species. Those forms that produce straight, non-spiral-forming sporophores will give rise to straight or slightly bent and wavy, long or short sporophores on all media. Upon reaching maturity, many of the types produce spirals on media favoring the formation of aerial mycelium. Other types vary in this respect, forming spirals on some media and not on others. There may even be variation within the same culture. Synthetic media usually give the most constant morphological characters. Many species that do not form aerial mycelium on organic media will do so on synthetic media.

Drechsler (97) suggested that the nature of the curvature of the spirals can be utilized as a distinguishing character. Krassilnikov, however, observed that most forms turn counter-clockwise (the reverse under the microscope), and only few in the reverse order. The nature of the medium is of great importance in this connection, thus making this character of doubtful taxonomic significance.

The fragmentation spores or the true conidia of members of the genus *Streptomyces* are spherical, oval, and elongated, whereas the segmentation spores or the oidiospores, characteristic of the *Nocardia,* are

Fig. 5 b (*see* p. 18).

usually cylindrical. The nature of these spores, especially the elongated ones, varies only occasionally. The manner of sporulation is constant. Because of these properties, the morphological characters form the most reliable basis for the separation of these organisms.

Cultural characteristics.—The growth and reactions of actinomycetes in culture media have been utilized most extensively for characterizing the individual species. There is, in this respect, however, considerable overlapping among the different forms, and one is frequently at a loss to know where to place a freshly isolated culture. Among the most important cultural properties are the following:

1. Shape and structure of colony, nature of vegetative growth, and appearance of aerial mycelium.
2. Anaerobism vs. aerobism, a very unstable property that cannot be sharply

defined, especially because of the frequent adaptation of anaerobes when freshly isolated to an aerobic form of life upon continued cultivation.

3. Proteolysis vs. non-proteolysis, such as gelatin liquefaction, milk coagulation and proteolysis, serum and egg albumen proteolysis, properties that are quantitative rather than qualitative in nature, with certain few exceptions.

4. Amylolytic v. non-amylolytic action, sucrose inversion vs. non-inversion, lipolysis, etc., properties that also cover phenomena which are largely adaptive in nature but that are valuable as secondary characteristics.

5. Thermophilic vs. mesophilic forms, a phenomenon which is also subject to adaptation and which cannot be very sharply defined because of the many intermediary types.

6. Pigment production, one of the most significant properties. Both endopigments and exopigments produced on synthetic and on organic media are given con-

Fig. 5 c (*see* p. 18).

sideration. Because of this, a number of descriptions have been based largely upon this property, beginning with the early differentiation between chromogenesis and non-chromogenesis on organic media. On synthetic media, many pigments are produced which resulted in designation of many forms on the basis of the pigment, whether present in the vegetative or in the aerial mycelium or whether it is dissolved in the medium. These pigments vary greatly in nature and intensity with the composition of different media, as well as with conditions of growth and age of the culture. Even with these limitations, however, pigment production is one of the most important and most easily recognizable characteristics, especially when media of known composition and definite conditions of culture are used.

7. Serum diagnosis. This may form a basis for more detailed differentiation of specific types. Aoki (11) established that agglutination reactions can be carried out with actinomycetes as readily as with bacteria; at first he found that the anaerobic forms fall into one group and the aerobic forms into 5 other groups; later, 3 more groups were added establishing in all 9 types. The complement fixation

reaction corresponded well to the agglutination reaction. The agglutinating receptors were present more abundantly in the spores than in the mycelium. V. Magnus (282) has been able to separate various strains of actinomycetes, on a serological basis into acid producers, alkali producers and neutrals; hemoagglutination phenomena were found to occur among 80 per cent of the acid producers.

8. Phage specificity. Certain actinomycetes are subject to attack by specific phages; thus, one actinophage attacks only the streptomycin-producing strains of S. *griseus*, and not others.

Biochemical characteristics.—This group of properties comprises quantitative rather than qualitative differences. The S. *coelicolor* group, for example, was found (78) to include forms which differ greatly in type of pigment produced.

Fig. 5 d (*see* p. 18).

On the basis of the reduction of nitrate to nitrite, the actinomycetes have been divided (134) into three groups: (*a*) those that give little or no reduction; (*b*) those that give moderate reduction; (*c*) those that give strong reduction. A similar basis of separation might be suggested for the properties of proteolysis, amylolytic action, and sucrose inversion. The ability to utilize specific carbohydrates is another biochemical property characterizing different types of organisms.

On the basis of these various properties, one may feel justified in establishing distinct species within the various genera.

Chapter II

IDENTIFICATION AND DESCRIPTIONS OF IMPORTANT TYPES

Classification of Actinomycetales:—The following classification of the actinomycetes is based entirely upon material included in BERGEY'S Manual of Determinative Bacteriology (34). For more detailed information as well as for literature references, the reader is referred to that Manual.

Order **Actinomycetales**

Organisms forming elongated, usually filamentous cells, with definite tendency to branching; hyphae not exceeding 1.5μ in diameter, mostly about 1μ or less. Usually producing a characteristic branched mycelium. Multiply by means of special spores, as well as by oidiospores or by conidia. The special spores are formed by fragmentation of the plasma within the spore-bearing hyphae, the latter being straight or spiral-shaped. The oidiospores are formed by segmentation, or by simple division of hyphae by means of transverse walls, in a manner similar to the formation of oidia among the true fungi. The conidia are produced singly, at the end of special, simple or branching conidiophores. They grow readily on artificial media and form well developed colonies. The surface of the colony may become covered with aerial mycelium. Some of the organisms are colorless or white, whereas others form a variety of pigments. They are either saprophytic or parasitic. In relation to temperature, most are mesophilic, though some are thermophilic. Certain forms are capable of growing at low oxygen tension.

Key to the families of order Actinomycetales:—

A. Mycelium rudimentary or absent, no spores formed—Family *Mycobacteriaceae.*
   I. Acid-fast organisms ..... *Mycobacterium.*
B. True mycelium produced:
   I. Vegetative mycelium divided by segmentation into bacillary or coccoid elements..... Family *Actinomycetaceae.*
      1. Anaerobic or microaerophilic, usually parasitic, non-acid-fast *Actinomyces.*
         Type species—*Actinomyces bovis.*
      2. Aerobic, partially acid-fast or non-acid-fast..... *Nocardia.*
         Type species—*Nocardia farcinica.*
   II. Vegetative mycelium normally remaining undivided—Family *Streptomycetaceae.*

(a) Conidia produced in chains, in aerial hyphae.....*Streptomyces*.
Type species—*Streptomyces albus*.
(b) Conidia produced terminally and singly on short conidiophores
Micromonospora.
Type species—*Micromonospora chalcea*.

Genus I. **Actinomyces** Harz

(*Streptothrix* Cohn; *Nocardia* Toni and Trevisan; *Cladothrix* Eppinger, Wolf-Israel fungus; *Anaeromyces* Castellani; *Brevistreptothrix* Lignières; *Cohnistreptothrix* Pinoy).

Actinomyces bovis Harz. (*Discomyces bovis* Rivolta; *Bacterium actinocladothrix* Afanasiev; *Nocardia actinomyces* Trevisan; *Actinomyces hominis* Wolf and Israel; *Streptothrix actinomyces* Rossi-Doria; *Cladothrix bovis* Macé; *Oospora bovis* Sauvageau and Radais; *Actinomyces bovis sulphureus* Gasperini; *Nocardia bovis* Blanchard; *Streptothrix israeli* Kruse; *Cladothrix actinomyces* Macé; *Actinomyces israeli* Lachner-Sandoval; *Streptothrix actinomycotica* Foulerton; *Streptothrix bovis communis* Foulerton; *Streptothrix bovis* Chester; *Discomyces israeli* Gedoelst; *Actinomyces sulphureus* Sanfelice; *Streptothrix sulphurea* Caminiti; *Sphaerotilus bovis* Engler; *Actinobacterium israeli* Sampietro; *Cohnistreptothrix israeli* Pinoy; *Proactinomyces israeli* Negroni; *Actinomyces wolf-israel* and *Corynebacterium israeli* Lentze; *Proactinomyces bovis* Henrici; *Actinomyces israeli* Rosebury).

According to Baldacci (17), most of the cultures listed as *A. bovis* comprise forms which have also been designated as *A. albus*, *A. sulphureus*, etc. These include the four species or varieties of *A. bovis* created in 1894 by Gasperini, namely, *A. bovis sulphureus*, *A. bovis farcinicus*, *A. bovis albus*, and *A. bovis luteo-roseus*. Waksman's description of *A. bovis* (443) is said to be equivalent to *A. bovis sulphureus*. Baldacci recommends that this species be considered as *A. sulphureus* Gasperini. Baldacci further included among *A. bovis*, *Streptothrix hominis* Foulerton, *Streptothrix luteola* Foulerton, *Actinomyces bovis* Harz *fide* Waksman, *Actinomyces hominis* Waksman (sub. *A. hominis* Bostroem), *A. bovis* Harz *fide* Lignières.

Very sparse development of erect aerial hyphae in growths produced in an atmosphere of reduced oxygen tension. These hyphae are occasionally septate, but no definite spores are formed; aerial mycelium heavier than vegetative mycelium, one micron or even more in diameter. Arthrospores about 2μ long. Gram-positive. Acid-fast. The substrate mycelium is initially unicellular, and the branches may extend into long filaments, causing the colony to adhere to the medium, or may give rise more or less quickly to irregular segments and characteristic angular branching. The colonies exhibit a considerable degree of polymorphism, but no stable variants have been established. Liquid media are usually clear.

Compared with the aerobic actinomycetes, the anaerobic organisms

show little biochemical activity. They do not produce soluble pigments on protein media or insoluble pigments in their growth; they have no proteolytic action on egg- or serum-containing media; they do not usually clot and do not peptonize milk, and in fact, rarely grow on it at all; they seldom grow on gelatin, and when there is a little flaky growth the tubes when cooled (from the 37°C. necessary for incubation) are found not to have been liquefied; and they have little or no haemolytic action on blood broth or blood agar. Acid is formed from certain sugars: according to Slack (403) from glucose, maltose, mannitol, sucrose, and lactose; according to Negroni and Bonfiglioli (318) from glucose, galactose, lactose, fructose, maltose, affinose, sucrose, and xylose. Milk also becomes acid.

Compared with the human strains, the strains of bovine origin display, according to Erickson (113), cultural and morphological differences. Their colonies are smoother and softer in consistency and are not adherent to the medium. Growth is scantier. The mycelium undergoes fragmentation very rapidly, and extensive ramification is rare. No aerial hyphae have been found. A much greater degree of uniformity is evident in colony development. Occasional turbidity occurs in liquid media. These strains also show a lesser ability to ferment sugars. Source: Jaw of cattle, udder of swine, and man (dental scum, tonsilar crypts).

Further information on the morphology and physiology of this organism is given later (p. 43).

In the latest edition of Bergey's Manual, a second species is recognized, namely *A. israeli*, which occurs in human tissues and is said to be responsible for human actinomycotic infections.

Genus II. **Nocardia** Trevisan

(*Actinomyces* Gasperini, Schottmüller, Henrici and Gardner; *Cohnistreptothrix* Orskov; *Streptothrix* Kruse, Caminiti, Rossi-Doria, Silberschmidt; *Cladothrix* Eppinger; *Brevistreptothrix* Lignières; *Actinobacterium* Haas; *Actinocladothrix* Afanassiev; *Actinobacille* Lignières and Spitz; *Actinococcus* Beijerinck; *Mycococcus* Bokor; *Asteroides* Puntoni and Leonardi; *Proactinomyces* Jensen.)

Slender filaments or rods, frequently swollen and occasionally branched, forming mycelium which after reaching a certain size may give the appearance of bacterial growths. Shorter rods and coccoid forms are found in older cultures. Conidia not formed. The nocardias stain readily, occasionally showing a slight degree of acid-fastness. Aerobic. Gram-positive. The colonies are similar in gross appearance to those of the genus *Mycobacterium*. Paraffin, phenol, and m-cresol are frequently utilized as sources of energy.

In their early stages of growth on culture media (liquid or solid), the structure of a nocardia is similar to that of a streptomyces. Both form a typical mycelium: hyphae branch abundantly, the branching being true. The hyphae vary in diameter between 2.5μ and 1μ, most of

FIG. 6.—*Nocardia asteroides*, grown on potato glucose-beef extract agar, gram stain, × 100. (*Prepared by* LITTMAN of Armed Forces Institute of Pathology).

them measuring 0.7-0.8μ, according to the species. The mycelium is not septate. The further development of nocardias, however, differs from that of streptomyces cultures: the filaments soon form a transverse wall and the whole mycelium breaks up into regularly cylindrical short cells, then into coccoid cells. On fresh culture medium the coccoid cells germinate into the mycelium. The whole cycle in the development of nocardias continues 2 to 7 days. Most frequently the coccoid cells are formed on the third to the fifth day, but those of certain species (*Nocardia ruber*, for example) can be found as early as the second day.

Numerous chlamydospores are sometimes found in older cultures of *Nocardia*. They are formed in the same way as the chlamydospores in true fungi; the plasma inside the filaments of the mycelium condenses into elongated portions. In older cultures of *Nocardia* many coccoid cells are changed into "durable" forms. The latter are larger than the vegetative coccoid cells, and the plasma of these cells is thicker than the plasma of vegetative cells. On fresh media the so-called "durable" cells germinate like the spores of *Streptomyces*. They form 2 to 3 germ tubes. Besides the cells mentioned, numerous involution forms can often be found in older cultures of *Nocardia*. These cells are thin, regularly cylindrical or coccoid, and are often transformed into a series of spherical or elliptical ampules and a club-like form (2 to more than 3μ).

The multiplication of nocardias proceeds by fission, budding, and rarely by special spores. Budding occurs often. The buds are formed on the lateral surfaces of the cells; when they have reached a certain size, they fall off and develop into rod-shaped cells or filaments. The spores are formed by the breaking up of the cell plasm into separate portions, usually 3 to 5 in number. Every portion becomes rounded, covered with a membrane, and transforms into a spore; the membrane of the mother cell dissolves and disappears. The spores germinate in the same way as those of *Streptomyces;* they form germ tubes which develop into a mycelium.

The colonies of nocardias have a paste-like or mealy consistency and can be easily taken up with a platinum loop. They spread on the glass and occasionally render the broth turbid. The surface colonies are smooth, folding, or wrinkly. Typical nocardias never form an aerial mycelium, but there are cultures whose colonies are covered with a thin coating of short aerial hyphae, which break up into cylindrical oidiospores.

Many nocardias form pigments. Their colonies are of a blue, violet, red, yellow, and green color. More often the cultures are colorless. The color of the culture serves as a stable character.

Krassilnikov (234) divided the genus *Nocardia* into two groups:

1. Well developed aerial mycelium—substrate mycelium seldom produces cross walls; the threads break up into long thread-like rods; branches of aerial mycelium produce segmentation spores and oidiospores, the latter being cylindrical with sharp

FIG. 7.—*Nocardia asteroides*, grown on potato glucose-beef extract agar, bottom of colony, gram stain, × 975. (*Prepared by* LITTMAN of Armed Forces Institute of Pathology).

ends; no spirals of fruiting branches. This group is the same as Group B of Jensen.

2. Typical forms—mycelium develops only at early stages of growth, then breaks up into rod-shaped and coccoid bodies; smooth and rough colonies, dough-like consistency; usually do not form aerial mycelium; similar to bacterial colonies; aerial mycelium may form around colonies.

The genus *Nocardia* can also be divided into two groups on the basis of acid-fastness:

1. Partly acid-fast organisms, which are nonproteolytic, nondiastatic, and utilize paraffin; usually yellow, pink, or orange-red in color.
2. Non-acid-fast organisms, which are diastatic, largely proteolytic, and do not utilize paraffin; yellow, orange to black in color.

Type Species: *Nocardia farcinica* Trevisan. (*Streptothrix farcinica* Rossi-Doria; *Oospora farcinica* Sauvageau and Radais; *Actinomyces farcinicus* Gasperini; *Actinomyces bovis farcinicus* Gasperini; *Bacillus farcinicus* Gasperini; *Cladothrix farcinica* Macé; *Streptothrix farcini bovis* Kitt; *Streptothrix nocardii* Foulerton; *Discomyces farcinicus* Geodoelst; *Actinomyces nocardii* Buchanan, and many others).

Filaments 0.25μ in thickness, branched. Markedly acid-fast.
Gelatin colonies: Small, circular, transparent, glistening.
Gelatin stab: No liquefaction.
Agar colonies: Yellowish-white, irregular, refractive, filamentous.
Agar slant: Grayish to yellowish-white, surface roughened.
Broth: Clear, with granular sediment, often with gray pellicle.
Litmus milk: Unchanged.
Potato: Abundant, dull crumpled, whitish-yellow.
Nitrites not produced from nitrates.
No soluble pigment formed.
Proteolytic action absent.
Starch not hydrolyzed.
Aerobic, facultative.
Optimum temperature 37°C.

Conant and Rosebury (75) recently presented (Table 2) a summary of some of the salient features of different species of *Nocardia*.

Habitat: Associated with disease in cattle, resembling chronic tuberculosis. Transmissible to guinea pigs, cattle, and sheep but not to rabbits, dogs, horses, or monkeys.

The last edition of Bergey's Manual contains descriptions of 33 species, with a large number of additional species only incompletely described.

Genus III. **Streptomyces** Waksman and Henrici

(*Streptothrix* Cohn; not *Streptothrix* Corda; *Actinomyces* Harz; *Discomyces* Rivolta; *Actinocladothrix* Afanassiev; *Nocardia* Trevisan; *Micromyces* Gruber; not *Micromyces* Dangeard; *Actinobacterium* Haas; *Carteria* and *Carterii* Musgrave, Clegg and Polk; *Euactinomyces* Langeron).

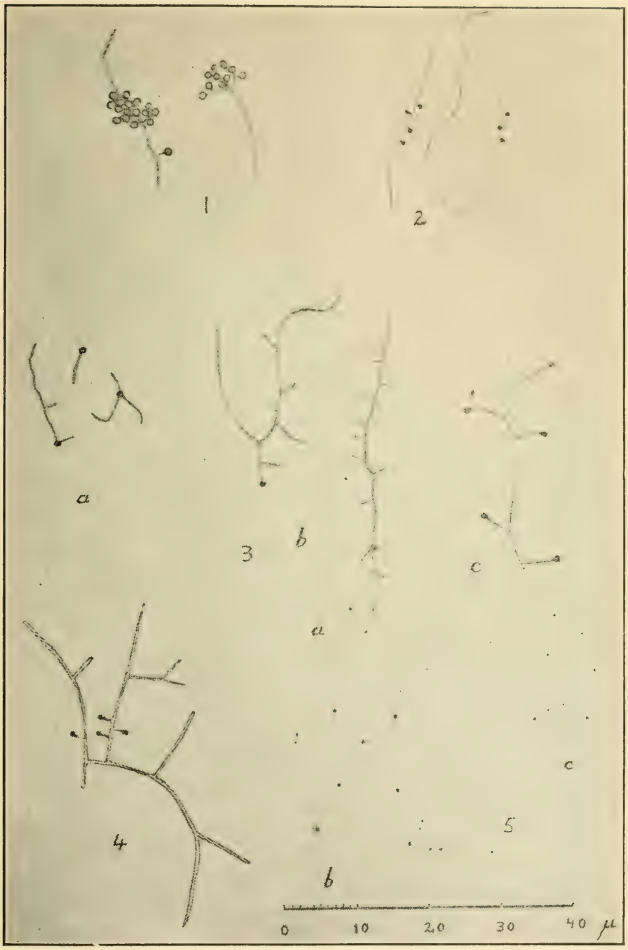

FIG. 8.—Branching and sporulation of different strains of *Micromonospora* (*from* JENSEN, 186).

TABLE 2: *Comparison of cultural, morphologic and staining reactions of species of Nocardia* (75):—

SPECIES	COLOR OF GRANULE	ACID-FAST	SABOURAUD'S GLUCOSE AGAR	CZAPEK'S AGAR (PIGMENT)	FRAGMENTATION OF MYCELIUM
1. *Nocardia asteroides* (Eppinger) Blanchard, 1896	Yellowish-white, with or without clubs	+	Glabrous, rarely chalky. Moist, soft, folded or wrinkled and granular. Yellow, orange-ochraceous, red	Yellow to orange	+
2. *Nocardia brasiliensis* (Lindenberg) Cast. and Chalmers, 1913	Yellowish-white, with or without clubs	+	Frequently chalky. Folded, cerebriform, tenacious and dry. Earthy odor. Yellow, orange-ochraceous	Yellow to orange ochraceous	+
3. *Nocardia madurae* (Vincent) Blanchard, 1896	Yellowish-white, with or without clubs	—	Glabrous. Moist, soft, wrinkled. Cream colored	Cream colored at first; later becoming pinkish to red	—
4. *Nocardia pellietieri* (Laveran) Pinoy, 1912	Red, with or without clubs	—	Small. Glabrous, heaped, wrinkled. Mucilaginous. Coral pink to red	Coral red	—
5. *Nocardia paraguayensis* (Almeida) Conant, 1947	Black, with clubs	—	Glabrous. Soft, white center. Projecting border adherent, darker	Dark cream	—

Organisms growing in the form of a much-branched mycelium with a typical aerial mycelium and spore formation. Aerobic. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in the animal body.

This genus can be divided, on the basis of the structure of sporulating hyphae into five groups:

GROUP 1: Straight sporulating hyphae, monopodial branching, never producing regular spirals.

GROUP 2: Spore-bearing hyphae arranged in clusters, or broom-shaped arising from compression of the sporophores.

GROUP 3: Spiral formation in aerial mycelium; long, open spirals.

GROUP 4: Spiral formation in aerial mycelium; short, compact spirals.

GROUP 5: Spore-bearing hyphae arranged on mycelium in whorls or tufts.

In group 5, the spore-bearing branches arise from definite knots, in the form of tufts or whorls, on one plane along the mycelium. These tufts consist of 3 to 10 sporophores and are formed more or less equidistant along the mycelium. This type of sporulation is ordinarily produced only on certain media, usually synthetic agar, but not on organic media.

Type Species: *Streptomyces albus* (Rossi-Doria em. Krainsky) Waksman and Henrici. (*Streptothrix alba* Rossi-Doria; *Cladothrix alba* Macé; *Nocardia alba* Chalmers and Christopherson; *Cladothrix dichotoma* Macé; *Streptothrix foersteri* Gasperini; *Streptothrix* 2 and 3 Almquist; *Actinomyces saprophyticus* Gasperini; *Oospora doriae* Sauvageau et Radais; *Cladothrix liquefaciens* Hesse; *Cladothrix invulnerabilis* Acosta e Grande Rossi; *Actinomyces chromogenus* Gasperini; *Streptothrix nigra* Rossi-Doria; *Streptothrix gedanensis* I Scheele et Petruschky; *Streptothrix graminearum* Berestneff; *Actinomyces thermophilus* (Berestneff) Miehe; *Cladothrix odorifera* Rullmann; *Actinomyces chromogenes* Gasp. β *alba* Lehmann and Neumann; *Oospora* sp. Bodin; *Oospora alpha* Price-Jones; *Streptothrix leucea* Foulerton; *Streptothrix candida* Petruschky; *Streptothrix lathridii* Petruschky; *Streptothrix dassonvillei* Brocq-Rousseau; *Streptothrix pyogenes* Caminiti; *Actinomyces albus* Krainsky; *Actinomyces sanninii* Ciferri; *Actinomyces almquisti* Duché; *Actinomyces gougeroti* Duché, and numerous others).

This is one of the most widely distributed and most widely described types in nature. It produces no soluble pigment and abundant white aerial mycelium. Various strains isolated by different investigators have been variously described. The most complete recent study was made by Duché (98) and by Baldacci (20).

Vegetative hyphae: Branched, 1μ in diameter.
Aerial mycelium: Abundant white, 1.3 × 1.7μ, with abundant spore formation.
Pigment, soluble: None.
Aerobic.
Odor: Characteristic.
Gelatin: Liquefied, no soluble pigment.
Bouillon: Flaky growth on bottom with surface pellicle.
Milk: Peptonized after having become coagulated. Reaction becomes alkaline.
Carrots and other vegetables: Excellent growth.
Habitat: Dust, soil, grains, and straw.

The last edition of Bergey's Manual contains descriptions of 73 species of *Streptomyces*, and an additional large number of incompletely described species.

Genus IV. **Micromonospora** Orskov

(*Thermoactinomyces* Tsiklinsky).

Well developed, fine, nonseptated mycelium, 0.3-0.6μ in diameter. Grow well into the substrate, not forming a true aerial mycelium at any time. Multiply by means of conidia, produced singly at end of special

conidiophores, on surface of substrate mycelium. Conidiophores short and simple, branched, or produced in clusters. Strongly proteolytic and diastatic. Comprise mostly saprophytic forms. These organisms occur commonly in hot composted manure, in aerial dust, and in soil, in river and lake waters, and in river and lake bottoms. Many are thermophilic and can grow at 65°C.

Key to the species of the genus Micromonospora:—

I. Vigorously growing organisms, typically copious spore formation on dextrose-asparagine agar.
  A. Vegetative mycelium pale pink to deep orange, no typical soluble pigment. ..... 1. *Micromonospora chalcea.*
  B. Vegetative mycelium orange changing to brownish-black, brown soluble pigment. ..... 2. *Micromonospora fusca.*

II. Slowly and feebly growing organisms, with scant spore formation on dextrose-asparagine agar, no soluble pigment.
  A. Vegetative mycelium pale pink to pale orange..... 3. *Micromonospora parva.*
  B. Vegetative mycelium yellow to orange-red. ..... 4. *Micromonospora globosa.*
  C. Vegetative mycelium blue..... 5. *Micromonospora vulgaris.*

Type Species: *Micromonospora chalcea* (Foulerton) Orskov. (*Streptothrix chalcea* Foulerton; *Nocardia chalcea* Chalmers and Christopherson; *Actinomyces chalcea* Ford).

Formation of a unicellular mycelium which produces distally placed, singly situated spores. No aerial hyphae. No surface growth in liquid medium. The organism absolutely resists desiccation for at least 8 months. Comparison between the power of resistance of the mycelium and the spores, respectively, will no doubt present great difficulty, because it is almost impossible to ensure that the two constituents are actually detached. Otherwise, the mycelium is but slightly capable of germinating, which may be ascertained by inoculating a water-agar plate liberally with a mixture of mycelial threads and spores. Though virtually all the spores germinate, the mycelial threads have never been found to form new colonies.

According to Jensen, vegetative mycelium on dextrose-asparagine-agar is heavy, compact, raised, not spreading much into the medium. Spore layer well developed, moist and glistening, brownish-black to greenish-black, this color sometimes spreading through the whole mass of growth.

Liquid media: Growth in form of small, firm orange granules or flakes.
Starch: Starch is hydrolyzed.
Gelatin: Liquefied.
Milk: Digestion of milk with a faintly acid reaction, mostly after a previous coagulation.

Many strains invert saccharose. Some strains reduce nitrate to nitrite. Most strains decompose cellulose. Proteolytic action seems stronger in this than in the other species of this genus. Optimum temperature for growth, 30-35°C. Thermal death point of mycelium, 70°C. in 2 to 5 minutes. Spores resist 80°C. for 1 to 5 minutes.

Habitat: Soil, lake mud, and other substrates.

This genus could be subdivided on the basis of the relations of the organisms to temperature, since it includes a number of thermophilic forms which grow readily at 55°-65°C., mesophilic forms having their optimum temperature at 30°C., and organisms growing at low temperature in lakes. Each of these can be divided into three groups, based on the structure of the spore-bearing hyphae. Among the thermophilic forms, only representatives of the first group have so far been isolated in pure culture, although the existence of the other two groups has definitely been demonstrated in microscopic preparations. These are:

Group 1. Simple spore-bearing hyphae.
Group 2. Branching spore-bearing hyphae.
Group 3. Spore-bearing hyphae in clusters.

Description of Several Important Actinomycetes:—In view of the great economic importance of some of the actinomycetes, several species with unusual physiological properties or of great practical value are described in detail here.

Actinomyces bovis Harz.

A. bovis is an anaerobic pathogen. It's most recent description, under the name of *A. israeli*, is given by Rosebury (367). This work served as a basis for the following summary.

A. bovis is a gram-positive, branching, filamentous organism, non-acid-fast, and not producing spores. The hyphae are usually less than 1μ in diameter. In tissue sections made from the lesions of actinomycosis, the organism appears in the form of compact granules or colonies which are often visible to the naked eye. The granules are circular or irregular in outline, or may comprise several colonies of different size and shape which have grown together. Each granule consists of a dense mass which stains irregularly in hematoxylin-eosin preparations but takes the violet dye in sections stained by Gram's method. The ends of individual filaments may be seen around the periphery of the granule, or part of the periphery may be composed of the radially arranged hyaline clubs. These can be stained with eosin. They are several times wider than the filaments, which can sometimes be traced within the structure of the club.

In exudates from actinomycosis, certain sulfur granules make their appearance. These are irregularly spherical masses, varying in diame-

ter. They are soft and easily broken under light pressure, but they may occasionally be tough or even calcified. The crushed granule appears as a disorganized mass of irregular, bent and branching filaments, some of which may terminate in the characteristic clubs. In preparations fixed and stained by Gram's method the structure of the granule is lost, the clubs not showing and the picture being that of a mass of irregular bent gram-positive rods.

The morphology of the organism has been described as varying from a compact mass of branching mycelium of gram-positive filaments to a mass of short rods which may be evenly stained or granular, and which show no indication of branching. These differences were found to be associated with roughness or smoothness of the colony. Rough colonies, whether grown on an agar surface or in an agar shake culture or in broth, show regular branching; twig-like forms are, however, much more common than long filaments. Intermediate and smooth colonies give a picture resembling that of the diphtheria organism, with granular and polar-stained forms, and with suggestive evidence of branching. Some of the smooth colonies may be derived from rough and clearly branched forms by subculturing; they show evenly stained rods with no distinguishing characteristics. The rough and intermediate forms often show terminal swellings or "clubbed forms" similar to those of the diphtheria organism; but the true clubs do not appear in cultures.

White or grayish colonies up to about 1.5 mm. in diameter, are produced in glucose-agar shake cultures at 37°C. within 3 to 6 days. The rough strains grow in a zone about 5 mm. wide, the upper limit being 0.5 to 2 cm. below the surface of the agar. A few colonies may be present below or above this zone, but no growth takes place on the surface. Smooth strains show no zone of concentrated growth; the colonies are uniformly distributed from the bottom of the tube to a level 0.5 to 1 cm. from the surface, where growth terminates abruptly. When a colony of a rough strain is transferred with a capillary pipette to a slide, it is usually found to be tough and difficult to break up and emulsify; it shows the characteristic compact branched mycelium.

Rough strains grow in glucose broth at 37°C. as white or grayish masses up to about 5 mm. in diameter at the bottom of the tube, the medium itself remaining perfectly clear. They are often difficult to break up. Intermediate strains tend to grow as smaller particles or granules either at the bottom or along the side of the tube, or as viscid or flocculent masses, with little or no general turbidity. Smooth strains, however, may produce uniform turbidity with or without a viscid or granular sediment.

On glucose agar or on brain-heart agar, incubated anaerobically with 5 per cent CO_2 for 4 to 6 days, rough or intermediate strains of *A. bovis* produce white-grayish to yellowish colonies having a diameter of not more than 1 to 3 mm. These colonies usually adhere to the medium, so that they are hard to remove with an inoculating needle, often com-

ing away all in one piece. The smooth colonies resemble those of white staphylococci or diphtheroids. They are soft and easily broken and emulsified. It was recognized, however, that on anaerobic-CO_2 plates, the colonies are very different from those found on aerobic media; an occasional rough white colony may, on examination, turn out to be a streptococcus.

A summary of the morphological and cultural properties of the pathogenic forms as compared to those of the saprophytes is given in Table 3.

Table 3: *Comparison of the parasitic and saprophytic actinomycetes* (367):—

	Parasitic actinomycetes	Saprophytic actinomycetes
Natural habitat:	Mouth and throat of man and probably of cattle and other animals; obligate parasites; sometimes pathogenic.	Soil, grains and grasses; widely distributed in nature; some pathogenic species, but most forms are non-pathogenic.
Cellular morphology:	Branched mycelium, gram-positive, not acid-fast. Marked tendency to fragment into bacillary forms.	Branched mycelium, gram-positive; some are acid-fast. Generally little tendency to fragment into bacillary forms.
Character of growth:	Bacteria-like colonies without aerial hyphae; no spores; no pigments.	Colonies more mold-like, often with aerial hyphae and spores (conidia); many produce yellow, orange or black pigments.
Temperature requirements:	Optimum, 37°C.; no growth at 22°C.	Optimum usually 15–20°C.
Relation to oxygen:	Oxygen tolerance limited; generally fail to grow or grow poorly under aerobic conditions.	Aerobic; some forms do not grow anaerobically.
Metabolism:	Probably never proteolytic. Ferment carbohydrates with production of acid.	Many forms actively proteolytic; may utilize carbohydrates without acid production.
Species recognized:	One only: *Actinomyces bovis*. (Provisional; heterogeneous but not yet satisfactorily subdivided.)	Many, subdivided into several families.
Pathogenicity:	Causative agent of true actinomycosis in man and animals.	Occasional causes of an actinomycosis-like disease, very rare in man, and of tropical cutaneous mycetomas, *e.g.*, Madura disease.

Streptomyces griseus (Krainsky) Waksman and Henrici

S. griseus, as a typical *Streptomyces,* produces both a vegetative and an aerial mycelium. The former varies in thickness from 0.3 to 2μ (0.5-1.3μ). It is (64) well developed, coenocytic when young, and branched in a typical monopodial form; occasionally two or more branches grow from the same place on the main hyphae; no true septa have been observed in the young vegetative mycelium, but are found in the older mycelium, and especially in the sporulating hyphae. The aerial mycelium is at first whitish, but later changes upon sporulation to yellowish green, with varying shades of cream, gray, buff, and brownish, depending on strain of organism and culture medium. The sporogenous hyphae may be borne directly upon the vegetative mycelium, several filaments arising from the same vegetative hyphae. Good sporulating strains produce straight, well branched sporogenous hyphae.

The spores are produced exogenously in chains on the aerial mycelium, over 200 spores having been counted (64) in a single chain of 3-day old cultures. The aerial sporogenous hyphae are often clavate and are continuous; transverse septae are laid down simultaneously, dividing the hypha into mononucleate or multinucleate segments. The cells between the septae increase in size, constrictions appearing at the septae, the spores being held in chains and in connection with each other by narrow fragile bridges. The spores vary in shape from spherical to cylindrical and in size from 0.7 to 0.9 × 0.7-1.9μ, the variations being observed in the same chain, as shown in Fig. 13.

The spores germinate at one or both ends, usually at the previous points of attachment to other spores. The germ tubes elongate by apical growth, the spore contents passing into it. The resulting mycelium branches and later leads to the formation of reproductive mycelium. A nucleus has been demonstrated in the spores, germ tubes, and young mycelium (64). The nuclei move with the cytoplasm. The spores are mononucleate or multinucleate.

The cultural characteristics of this organism have been (34) briefly described as follows:

Gelatin stab: Greenish-yellow or cream-colored surface growth with brownish tinge. Rapid liquefaction.

Synthetic agar: Thin, colorless, spreading, becoming olive-buff. Aerial mycelium thick, powdery, water-green.

Starch agar: Thin, spreading, transparent.

Dextrose agar: Elevated in center, radiate, cream-colored to orange, erose margin.

Plain agar: Abundant, cream-colored, almost transparent.

Dextrose broth: Abundant, yellowish pellicle with greenish tinge, much folded.

Litmus milk: Cream-colored ring; coagulated with rapid peptonization, becoming alkaline.

Potato: Yellowish, wrinkled.

Nitrites produced from nitrates.

Proteolytic action in milk and gelatin.

The pigment formed is not soluble.

Starch is hydrolyzed.

Aerobic.

Optimum temperature 37°C. The optimum temperature for certain physiological reactions is much lower; for example, 25° to 28° for streptomycin production.

Habitat: Peat, soils, river flats, and dust. Numerous strains of this organism have been isolated from habitats which range from the throat of a chicken to that of rich garden soils and cultivated peats.

S. griseus represents a most variable group of organisms. This is brought out quite emphatically in an examination of the ability of different strains within this species for their ability to produce antibiotic substances. These have been classified into 4 groups.

1. Those strains which produce streptomycin, the amount of antibiotic produced varying greatly with the individual strains under different conditions of culture; they are sensitive to actinophage.
2. Those strains which produce only or predominantly grisein or grisein-like substances; they are resistant to actinophage.
3. Those strains which produce other antibiotics, which are active against gram-positive bacteria only, and the exact nature of which is still unknown.
4. Those strains which produce no antibiotic at all.

Another very important characteristic of S. *griseus* strains is their ability to produce mutants. So far, 2 mutants have been isolated from the streptomycin-producing cultures: (*a*). A colorless mutant, producing no aerial mycelium, not producing any streptomycin and sensitive to this antibiotic as brought out in TABLE 4. (*b*). A pigmented mutant, producing pink to vinaceous colored vegetative growth, but forming the

TABLE 4: *Streptomycin production and streptomycin sensitivity of different strains of S. griseus and their variants* (395):—

STRAIN OR VARIANT	ORIGIN	PRODUCTION OF STREPTOMYCIN*	STREPTOMYCIN SENSITIVITY**
Strain No. 4	Sporulating active form	38	>3,125
Strain No. 19	Sporulating active form	128	>3,125
Variant 3	Non-sporulating form	0	20
Variant 4	Non-sporulating form	0	16
Variant 6	Non-sporulating form	4	27
Reverted strain	Sporulating active form	37	>3,125

* Units of streptomycin in 12-day cultures.

** Units of streptomycin required to inhibit growth of particular strain or variant in 1 ml of medium.

typical aerial mycelium; this mutant forms no streptomycin but another antibiotic which is not active against gram-negative bacteria.

The life cycle of S. *griseus* in relation to the production of streptomycin has been described (481) as follows:

The growth of S. *griseus* reaches a maximum in stationary cultures in 10 days and in submerged cultures in 3 to 5 days, followed by the lysis of the mycelium. Growth is accompanied by a gradual rise in the *p*H value of the culture, and in the ammonia and amino nitrogen contents. The total nitrogen in the mycelium tends to be higher during the active stages of growth. The production and accumulation of streptomycin parallels the growth of the organism. After maximum activity has been reached, there is a drop in activity, which is rapid in submerged cultures. For the production of streptomycin, the presence in the medium of an organic substance is required. This substance may either serve as the precursor of the streptomycin molecule as a whole or of an important group in the molecule, or it may function as a prosthetic group in the mechanism essential for the synthesis of the streptomycin. Such a factor can gradually be synthesized by the organism, when it is provided in the medium in a preformed state, however, as in meat extract or in corn steep liquor, the process of streptomycin synthesis is greatly facilitated. Streptomycin is also formed in purely synthetic media.

In addition to the streptomycin complex, S. *griseus* produces at least 2 other antibiotics one of which, actidione, is active only against fungi, and another, streptocin, which is present in a limited amount in the culture filtrate but more abundantly in the mycelium. Streptocin is soluble in organic solvents and is not active against gram-negative bacteria.

Streptomyces lavendulae (Waksman and Curtis) Waksman and Henrici

S. *lavendulae* also represents a large heterogeneous group of organisms which differ greatly in some of their biochemical properties, notably the production of antibiotic substances.

The first culture of S. *lavendulae* was isolated from a New Jersey soil in 1915 (460). Its early description was given (34) as follows:

Mycelium and hyphae coarse, branching. Spirals close, 5 to 8μ in diameter. Conidia oval, 1.0 to 1.2 by 1.6 to 2.0μ.

Gelatin stab: Creamy to brownish surface growth. Liquefied.

Synthetic agar: Thin, spreading, colorless. Aerial mycelium cottony, white, becoming vinous-lavender.

Starch agar: Restricted, glistening, transparent.

Plain agar: Gray, wrinkled.

Dextrose broth: Abundant, flaky sediment.

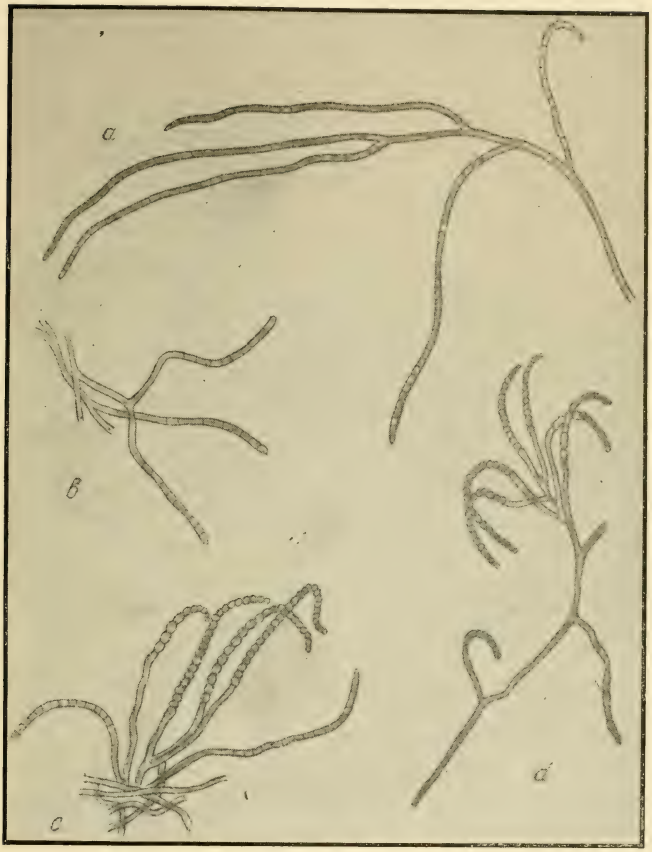

Fig. 9.–Sporulation of straight aerial hyphae of species of *Streptomyces* (*from* Krassilnikov, 234).

Litmus milk: Cream-colored ring. No coagulation; peptonized, with strong alkaline reaction.
Potato: Thin, wrinkled, cream-colored to yellowish.
Nitrites produced from nitrates.
Soluble brown pigment formed.
Peptonization of milk and gelatin.
Starch is hydrolyzed.
Aerobic.
Optimum temperature 37°C.
Habitat: Widely distributed in soils and other natural substrates.

The first antibiotic produced by S. *lavendulae* was designated as streptothricin (452). Since then, several other antibiotics have been isolated from members of this group. Some of these antibiotics, notably, lavendulin, streptolin and streptothricin VI, are similar to streptothricin in their general antimicrobial spectra, but they differ in their quantitative effects upon different bacteria, and in their greater or lower toxicity to animals. Some of the antibiotics produced by organisms belonging or closely related to the S. *lavendulae* group are distinctly different from streptothricin both chemically and in their antibiotic spectra, as is the case for chloromycetin.

The streptothricin-producing S. *lavendulae* strains give rise to a number of variants (478). Some of these variants produce on glucose-peptone a blue diffusible pigment; others form a brown pigment. The vegetative mycelium of the blue pigment-forming variants is pale-blue with scattered, small pin-point areas of deep blue. Upon complete sporulation, the vegetative growth is covered with thick lavender-colored aerial mycelium; occasional sunken areas are of a slightly bluish tinge, these areas corresponding to the pin-point regions of the deeper blue. The under surface of the vegetative growth is cream-colored except for the small blue spots. The other variants produce a colorless to cream-colored vegetative growth free of any blue pigment whatsoever; one to two days later, a brown diffusible pigment appears, the growth becoming covered with abundant lavender-colored mycelium. On subsequent transfer on fungus-agar slants, the two types of variants prove to be rather stable.

Some of the strains isolated from an active streptothricin-producing culture may lose the property of producing this antibiotic. Other strains may form antibiotics which vary from the typical streptothricin either in their antibacterial spectra or in their toxicity to animals.

In a comparative study of the relation between growth of the organism and production of antibiotic, it was found (452) that both in stationary and in shaken cultures growth and activity reach a maximum and then decline, the maximum for the first preceding somewhat that of the second. Since the nitrogen in the dry mycelium varies between 7 and 9 per cent, growth may be expressed in terms of the dry weight of the mycelium or in terms of its nitrogen content. It must be concluded, therefore, that production of streptothricin is not a result of

autolysis of the mycelium but is due to cell nutrition or to cell synthesis. This renders the mechanism of the production of this substance distinct from that of tyrothricin, for example, which is a result of autolysis of the bacterial cells, or of penicillin, which is produced at a much later stage of growth of the organism, that is, when it reaches an alkaline reaction.

The efficiency of utilization of carbon and nitrogen by S. *lavendulae* is very high. At the maximum growth stage, 65 per cent of the nitrogen in the glycine added to the medium was found to be converted into actinomyces cell substance. Since as much as 330-350 mg. of mycelial growth was obtained from 1 gm. of raw starch, the efficiency of utilization of the carbon, considering the carbon content of the starch as well as of the glycine, is about 40 per cent.

Streptomyces venezuelae Ehrlich, Gottlieb, Burkholder, Anderson and Pridham

S. *venezuelae,* the organism that produces chloromycetin (chloramphenicol) was isolated from two different soils, one a mulched field near Caracas, Venezuela, and the other a compost soil at Urbana, Illinois (103, 103*a*).

Primary mycelium growing in agar substrates is thin-walled, colorless, hyaline, monopodially branched. Mature vegetative hyphae vary in diameter from 0.9 to 1.8μ and the branches grow to about 150μ in length. Sometimes the substratal mycelium forms oval spores by fragmentation. The aerial mycelium is lavender under the microscope, thick-walled, generally not much branched, straight or slightly and irregularly curved, not forming spirals, having individual filaments that appear stiff, and arising frequently from the primary mycelium at the surface of the substrate. Individual filaments are rarely septate, are 1.0 to 1.8μ in diameter, and vary in length up to about 350μ. In young colonies, the aerial hyphae project outward radially over the surface of the colony and show a lavender color when examined microscopically. The color of colonies when viewed on agar without magnification is gray to light tan or pink, but not lavender. Distal portions of the aerial hyphae commonly subdivide into unbranched oidial spore chains, which are readily fragmented into small groups or individual spores.

The spores are oval to oblong. Mature spores range from about 0.4 to 0.9μ in diameter and from 0.7 to 1.6μ in length. The spores formed by fragmentation of hyphae in the substrate are generally smaller than those formed from the aerial hyphae. Individual spores are colorless at maturity but in mass appear tan to gray when viewed without magnification. They may be stained readily with crystal violet

and other bacteriological dyes. The spores are uninucleate, as determined by Giemsa staining.

The two strains of S. *venezuelae* were similar, in their cultural and physiological properties, to S. *lavendulae*, although they differed from S. *lavendulae* in their ability to utilize various carbohydrates. The former utilized arabinose, rhamnose, xylose, lactose and fructose. The utilization of these by S. *lavendulae* was either negative or questionable. The two strains also differed from S. *lavendulae* in their sensitivity to actinophage and in serological reactions.

Streptomyces antibioticus (Waksman and Woodruff) Waksman and Henrici

A detailed description of S. *antibioticus* has been given by Waksman and Woodruff (491).

Morphology: Spore-bearing hyphae produced in the form of straight aerial mycelium. The sporophores are arranged in clusters; no spirals formed. The spores are nearly spherical to somewhat elliptical.

Gelatin: Dark brown growth on surface, with patches of gray aerial mycelium. Dark pigment produced, which gradually diffuses into the unliquefied part of gelatin. Liquefaction of gelatin at first very slow, later becoming rapid.

Potato plug: Folded, brown-colored growth, with a thin black ring on plug, fading into a bluish tinge. No aerial mycelium.

Carrot plug: Cream-colored to faint brownish growth. No aerial mycelium. No pigment.

Litmus milk: Thick, brownish ring on surface of milk. Mouse-gray aerial mycelium with greenish tinge; growth becomes brown, especially in drier portions adhering to glass. No reaction change, no coagulation of milk, no clearing; whitish sediment at bottom of tube. Old cultures—heavy growth ring on surface of milk, heavy precipitation on bottom; liquid brownish to black in upper portion.

Czapek's agar: Thin, whitish growth. Thin, gray aerial mycelium.

Peptone media: Production of dark pigment at early stage of growth is very characteristic. Growth brownish, thin, with yellowish-gray to yellowish-green aerial mycelium.

Odor production: Very characteristic soil odor.

Antagonistic properties: Has a marked antagonistic effect on gram-positive and gram-negative bacteria (much more so on the former than on the latter), as well as on actinomycetes. It is also active against fungi, which vary in degree of sensitivity.

Habitat: Found in soil. Isolated on *Escherichia coli* washed agar plate, using living cells of *E. coli* as the only source of available nutrients.

Streptomyces aureofaciens Duggar

S. *aureofaciens*, the organism that produces aureomycin, was isolated from the soil (99*a*).

Pigment production (golden yellow) is well developed in most strains of this organism grown on meat extract-asparagine-glucose agar, or on potato-dextrose agar, and on potato plugs. The substrate mycelium of young colonies is hyaline at first, commonly becoming yellow in 2 to 3 days. The aerial mycelium is white. The first-formed spores are white, but the entire heavily sporing surface of a slanted agar culture gradually changes in 5 to 7 days at 28°C. through brownish gray to a dark, drab gray. At the same time most of the substrate mycelial color disappears. The reverse color of slants at its best is golden tan, later tawny.

Aureomycin is a weakly basic compound which contains both nitrogen and nonionic chlorine. Aureomycin when treated with alcoholic ferric chloride gives a greenish-brown color by reflected light and reddish color by transmitted light. The crystalline free base has the following properties: m.p., 168-169°C; solubility in water, 0.5-0.6 mg/ml at 25°C; soluble in the cellosolves, dioxane, and carbitol; slightly soluble in methanol, ethanol, butanol, acetone, ethyl acetate, and benzene; insoluble in ether and petroleum ether; very soluble in aqueous solution above *p*H 8.5.

Streptomyces scabies (Thaxter) Waksman and Henrici

Morphology: wavy or slightly curved mycelium, with long branched aerial hyphae, showing a few spirals. Conidia more or less cylindrical; 0.8 to 1.0 by 1.2 to 1.5μ.

Gelatin stab: Cream-colored surface growth, becoming brown. Slow liquefaction.

Synthetic agar: Abundant, cream-colored, wrinkled, raised. Aerial mycelium white, scarce.

Starch agar: Thin, transparent, spreading.

Dextrose agar: Restricted, folded, cream-colored, entire.

Plain agar: Circular, entire colonies, smooth, becoming raised, lichenoid, wrinkled, white to straw-colored, opalescent to opaque.

Dextrose broth: Ring in form of small colonies, settling to the bottom.

Litmus milk: Brown ring with greenish tinge; coagulated; peptonized with alkaline reaction.

Potato: Gray, opalescent, becoming black, wrinkled.

Nitrites produced from nitrates.

Brown soluble pigment formed.

Peptonization of milk and gelatin.

Starch is hydrolyzed.

Aerobic.

Optimum temperature: 37°C.

Habitat: Soil; cause of potato scab.

This is a large heterogeneous group of organisms, occurring in nature in the form of many strains. A number of specific organisms, said to be

FIG. 10.—*Streptomyces venezuelae*, grown on potato glucose-beef extract agar, gram stain, × 975. (*Prepared by* LITTMAN of Armed Forces Institute of Pathology).

causative agents of scab, have been described. Because of lack of experimental demonstration, it is difficult to state how many of these actually cause scab. The ease with which numerous saprophytic actinomycetes are isolated from the surface of material that has been in contact with the soil justifies these doubts.

In a study of the effect of environmental conditions upon the growth of S. *scabies,* the following conclusions were reached:

S. *scabies* grows within a wide range of temperature (8° to 38° C.). Good growth and maturity occur between 13° and 32°C., and the optimum temperature is about 27°C. Therefore, under average field conditions in most potato growing areas, it appears that temperature, as it affects host and pathogen only, cannot be a very important factor in the scab problem. The spores survive temperatures up to 90°C. (moist heat) for ten minutes.

S. *scabies* is a strong aerobe. The spores will germinate with an extremely small supply of oxygen, but a large amount is required for subsequent development. Maturity, as indicated by dark aerial hyphae, will not take place in the absence of oxygen. Amount of oxygen, not partial pressure, is the limiting factor for germination and growth.

It was found that the germination of spores of S. *scabies* on nutrient agar was greatly retarded by a lagging film of excess water. The inoculum of S. *scabies* appeared to increase most rapidly at a soil moisture content about optimum for plant growth.

The limiting acid reaction for germination of the spores of the strain of S. *scabies* used was found to be about *p*H 5.3. Germination occurred most quickly at about *p*H 8.5, and an optimum development took place at this point. Because of the higher *p*H of the tuber and a strong tendency of the pathogen to make its habitat (scab pustule) alkaline, severe scab may be expected in soils ranging from a strongly alkaline reaction to at least *p*H 5.4.

Chapter III

MORPHOLOGY AND LIFE CYCLE

Lack of complete understanding of the distinct morphological characteristics of the actinomycetes and of their mode of reproduction has been one of the major causes of the existing confusion concerning the nature and systematic position of this group of microorganisms. The fact that some species of actinomycetes resemble the true fungi in many respects whereas other species resemble the true bacteria more closely, and the fact that actinomycetes are characterized by marked variation in morphology and in cultural characteristics, especially when grown on artificial media, have also contributed to the confusion.

One of the early students of the actinomycetes, F. Cohn, recorded in 1875 that the "ray fungi," a common designation given to this group of organisms, are fungus-like in nature. This point of view was held by a number of subsequent investigators, notably Thaxter in 1891, Lachner-Sandoval in 1898, Berestnew in 1899, Neukirch in 1902, and more recently Drechsler, Orskov, Jensen, and others. The production of a very fine mycelium consisting of unicellular branching hyphae definitely emphasized their similarity to the true fungi.

On the other hand, the unicellular nature of the mycelium, its very fine structure, the resemblance in dimensions of the hyphae and of the spores to those of the bacteria, and the appearance of stained preparations prepared in accordance with bacteriological practice—all tend to suggest that one is dealing here either with bacteria or with bacteria-like organisms. Since most of the early and even the more recent investigators cultivated the actinomycetes on complex organic media, the differences in morphological structures and cultural characteristics tended to be obscured. Marked differences became apparent only with the introduction of synthetic media for the growth of actinomycetes and with the development of suitable microscopic techniques for examination of these organisms in an undisturbed state. It has become recognized that the actinomycetes possess morphological properties which not only are distinct from those of the bacteria but which are, within certain limits, fairly constant.

Staining of Actinomycetes:—In addition to the direct methods of examining the structure of the actinomycete colonies and their growth characteristics, that is, the general methods developed by students of

FIG. 11.—*Streptomyces* sp., grown on potato glucose-beef agar, gram stain, × 975. (*Prepared by* LITTMAN of Armed Forces Institute of Pathology).

fungi and bacteria, certain special methods have also found application. Among these, it is sufficient to mention the following:

1. *Method of Henrici.*—A drop of melted agar medium is placed on a slide, allowed to cool somewhat, and inoculated with the actinomyces culture. The agar is then spread in a thin film on the slide. The agar may also be allowed to cool first before being inoculated with a sharp needle. The slide is then incubated in a sterile moist chamber. After growth has taken place, the slides are allowed to dry, are fixed in alcohol, and stained. The entire colony, with both vegetative and aerial mycelium can thus be stained and examined in an undisturbed condition.

2. *Method of Drechsler.*—The culture is grown on a synthetic medium, and the fully developed colony is cut from the agar as carefully as possible. A slide smeared with albumin fixative is brought into firm contact with the surface mycelium of the colony, then separated from it, precautions being taken to avoid any sliding of the two surfaces on each other. If the growth is not too young, the upper portions of the aerial mycelium will be left adhering to the slide without much disarrangement. The adhered growth is then killed and fixed at once, and the preparation is stained and mounted in balsam. Preparations in which the spore chains have commenced to disintegrate are impaired by the large masses of free spores. The most convenient fixative agent is 95 per cent alcohol. As a stain, Haidenhain's iron-alum haemotoxylin is good for protoplasmic structures. Delafield's haemotoxylin, allowed to act for 24 hours with the proper degree of decolorization, yields deeply stained, clear preparations showing distinctly the various mycelial structures of the organism.

3. *Other methods.*—Various special methods have been utilized for preparing actinomyces cultures for staining. It is sufficient to mention the use of a drop of liquid synthetic medium placed on a cover slip, which is then inoculated with a few actinomyces spores and incubated in a sterile moist chamber or in the form of a hanging drop preparation. The liquid medium may also be allowed to flow around an actinomyces colony, which has been removed from an agar plate and placed on a cover slip; the peripheral growth may be stained.

All actinomycetes are gram-positive, although certain thermophilic forms, according to Lieske, may be gram-negative at temperatures above 50°C.

Most actinomycetes are non-acid-fast. Some of the *Nocardia* species, however, especially many of the pathogenic forms, are acid-fast (145, 432).

The mycelium stains uniformly, except in older cultures. The presence of metachromatic granules has been observed by Brussov (49) and Drechsler (97); this was believed by some investigators to indicate that the cultures possess the property of pleomorphism. The granules in the mycelium can be readily stained with methylene blue (1:1,000) and decolorized by sulfuric acid. Droplets of fat, often pigmented, can be seen frequently in the mycelium (242). The formation of vacuoles has also been reported (97). Neukirch (321) differentiated the ectoplasm of the actinomycetes from the endoplasm, on the basis of staining with dilute methylene blue, the first being dark blue and the second light blue.

The presence of nuclei in actinomycetes has aroused considerable

discussion. The presence of fine grains in preparations treated with dilute methylene blue was looked upon as substantial evidence of the presence of a nucleus (321). Others considered these granules, however, as merely fatty bodies or of a metachromatic nature. Krassilnikov submitted evidence that these granules consist of chromatin substance, which plays the role of a nucleus. Young hyphae contain single grains which are larger in older cultures and could be distinguished only with difficulty from the rest of the protoplasm.

By use of the Feulgen reaction, von Plotho (340) demonstrated that the reacting substance is distributed in the protoplasm of the actinomycetes in all stages of its development. He reached the conclusion that nuclear substance is present in the cell plasma. This substance can become concentrated into special nuclear bodies, especially in the mature spores. The presence of thymonucleic acid bears evidence of this fact. These results are not in agreement with those obtained by Rippel (361), who believed that the bodies stained by the Feulgen reaction are fats in nature.

Jensen described (186) the staining reactions of *Micromonospora* as follows: The hyphae and spores stain easily with all the usual bacterial stains, such as carbol fuchsin, aqueous fuchsin, methylene blue, gentian violet. Delafield's haematoxylin gives fine and clear preparations, especially when material is fixed with sublimate alcohol. The spores stain more intensely than the hyphae. All the strains are gram-positive, but never acid-fast. Whether nuclei are present in the spores and mycelium was difficult to decide because of the minuteness of the objects. Preparations were stained by the method of Schumacher for demonstrating nuclear material. The preparation was dried on a slide and treated for 2 to 4 hours with 25 per cent hydrochloric acid, washed first with water, then for 10 seconds with dilute Na_2CO_3 solution, and finally stained for 30 seconds with carbol thionine. The presence of deeply stained minute granules was demonstrated in old spores, in germinating spores, and in young mycelium.

The nuclear method of staining bacteria was applied successfully to the staining of sporulating actinomycetes (222). It consists in fixing the cells with osmic acid in N HCl, and staining with the Giemsa stain. The acid is usually applied for 6 to 20 minutes at 55°C., and the stain, diluted 1 to 30, 5 to 30 minutes. The preparations are dehydrated with acetone and xylose and mounted in Canada balsam or in the weak staining solution. To show the cell boundaries, the osmic acid preparations are placed for 30 minutes in a 5 per cent aqueous solution of tannic acid, rinsed in water, stained for 2 to 4 minutes in crystal violet 1:10,000, and mounted in the stain or in water.

General Morphology:—

Colony formation.—Growth of an actinomyces on a solid or in a liquid medium results in the formation of a mass of growth usually

designated as a "colony." This is not a true colony in a bacterial sense, since it is not an accumulation of a number of cells originating from a single cell or from several similar cells. It is rather a mass of branching filaments which originated from a spore or from a bit of vegetative mycelium.

The actinomyces colony is made up often of two types of mycelium, consisting primarily of vegetative or substrate growth and of secondary aerial or sporogenous growth. These two types of mycelium often show fundamental differences in appearance, composition, and biological activities. The vegetative mycelium grows into the medium, whereas the aerial mycelium grows on the surface; the well-developed sporulating hyphae and the reproductive spores are produced in the aerial mycelium. Some actinomycetes form only the vegetative mycelium, whereas others produce both types.

Vegetative mycelium.—The vegetative growth of the actinomycetes, or the stroma, is usually shiny, gel-like, or lichnoid in appearance and varies in size, shape, and thickness. The color of the growth may be whitish or cream colored, as well as yellow, red, pink, orange, green, or brown. In addition to the insoluble pigments, certain water-soluble pigments are produced. Some of the pigments, notably the brown and the darker or chromogenic pigments are formed upon complex organic media and are a result of the action of certain enzymes of the tyrosinase type, which are able to oxidize some of the organic constituents of the medium to give the particular pigments. The red, yellow, and blue pigments are synthetic in nature.

When actinomycetes spores are inoculated into a fresh medium, they germinate rapidly, usually within 2 to 6 hours, and give rise to one or more germ tubes, as shown in Fig. 13. These grow into long hyphae or threads which gradually develop into a complex mycelium. The length and diameter of the hyphae differ considerably for the various organisms. Some are straight and long, reaching 600μ or more; others are only 50 to 100μ in length, and are much branched and curved. The vegetative mycelium varies in diameter from 0.2 to 0.8μ. Occasionally, involution forms are produced which have even a greater diameter. The structure of the hyphae also varies with the composition of the medium, the conditions of growth, especially temperature, and the presence of stimulating or injurious substances. On the basis of the length of the hyphae, Lieske (186) divided the actinomycetes into long-hyphal and short-hyphal forms. It is doubtful, however, whether such a sharp line of demarkation can be drawn for all organisms within this group and for all media upon which they are usually grown.

In older cultures, the vegetative mycelium becomes brittle and readily breaks into fragments of uneven length. The mycelial fragments are very small, usually 1μ or less in length. Together with the cellular contents they form a granular mass which deposits on the bottom of liquid cultures. Some cultures undergo rapid lysis, especially

at higher temperatures or when grown under submerged conditions. Others are subject to attack by specific phages. When inoculated into fresh medium, the finer or disintegrated particles give rise to a normal mycelium. Some investigators (158, 226) were lead to consider this phenomenon as symplasm formation, or as a stage in the life cycle of the actinomycetes. Krassilnikov (234) rejected this concept and emphasized the fact that it is not the symplasting mass as a whole but the sporulating bodies present within the lysed material that are responsible for the reproductive capacity of the organism.

Aerial mycelium.—Many of the actinomycetes, notably members of the genus *Streptomyces,* are capable of producing an aerial mycelium superimposed upon the vegetative growth. The production of the aerial mycelium by various actinomycetes depends on the culture, composition of medium, and conditions of incubation. These factors also influence the nature and abundance of the mycelium. The aerial hyphae vary considerably in length and may have a diameter of 1μ or even 1.4μ. Usually, they are short and straight or wavy and much branched. Some organisms produce long hyphae that are little-branched, straight, or slightly curved. The aerial mycelium may cover the whole colony either in the form of a cottony mass or as a powdery, chalk-like to almost granular layer. Certain organisms produce an aerial mycelium in the form of tufts or as concentric zones over the vegetative growth; in a few cases, it may be compacted into bodies resembling coremia, the central portion consisting of vegetative growth and the surface of aerial mycelium.

These sporulating hyphae represent a well-characterized sporogenous apparatus, consisting of a sterile axial filament bearing branches in an open racemose or dense capitate arrangement. The primary branches may function directly as sporogenous hyphae or may produce branches of the second and higher orders. In the latter case sporogenesis is confined to the terminal elements, and the hyphal portions below the points of attachment of branches remain sterile.

The morphology of the spore-bearing hyphae of the various actinomycetes exhibits distinct individuality and can readily serve as a basis for specific differentiation. The specialized, sporogenous hyphae are distinguished from the sterile hyphae of the aerial mycelium at an early stage of their development. Though the diameter of the sterile mycelium which arises through the elongation of the growing filament tip shows very little subsequent increase in thickness, the sporogenous hyphae are, in the beginning, thinner than the axial hyphae from which they are derived. Increase in thickness of the sporogenous hyphae follows after the final linear extension has been attained. The final diameter of the sporogenous hyphae is in most cases appreciably more than that of the vegetative hyphae.

The formation of the aerial from the vegetative mycelium has been ascribed (222) to agglomerations or fusion of filaments which give rise

to "initial cells." These are formed first in the center of the colony, then at the periphery. The "fusion cells" consist of darkly staining nuclear bodies surrounded by protoplasm and later enclosed by cell walls. They grow into the aerial mycelium by a process of sprouting and subdividing. Transverse septa are easily demonstrable in the aerial mycelium. The division of the nuclear cylinders in the cells of this mycelium initiates spore formation.

Some actinomycetes produce an aerial mycelium which has the form of "fairy rings." These consist of concentric spore-bearing rings and spore-free rings disposed in zones. It has been suggested that ring formation is a result of diffusion of injurious substances present or formed in the medium or that it is due to the action of light, which produces a change in transpiration and temperature. This phenomenon may be closely related to the autolytic reactions and attack of certain species by phage.

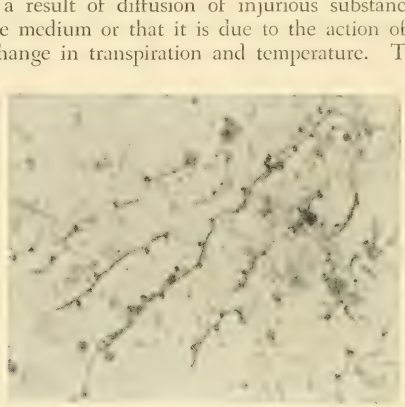

Fig. 12 a-d.—Different forms of sporulation of *Micromonospora* growing in composts, as shown by contact slide preparations (*from* Waksman, Cordon and Hulpoi, 459).—for b-d, *see* pp. 53-55.

The aerial mycelium is variously pigmented, from shades of white or gray, to yellow, orange, red, rose, lavender and green. The dry powdery appearance of the aerial mycelium of actinomycetes and the difficulty of wetting the spores appear to be due to the presence of lipids in their outer walls. These substances are removed by fat solvents and wetting agents and are destroyed by alkalies. Staining with Sudan IV distinguishes the lipid-containing aerial mycelium from the vegetative mycelium (114).

The manner of spore formation depends upon the specific nature of the organism and upon the conditions of cultivation. The conidiophores or sporophores produced on the aerial hyphae comprise several types, as pointed out previously (p. 30).

The composition of the medium is of great importance in influencing the manner of sporulation. Synthetic media are best for studying this phenomenon. The process of sporulation is favored by dryness, aerobic conditions, and carbohydrate nutrition.

Cytology.—The formation of cell walls by actinomycetes has aroused much speculation. Normally, growing mycelium does not show any cell wall; it becomes apparent, however, when the plasma constricts and breaks up into fractions. This can be seen either in old cultures or during the process of sporulation of the aerial mycelium. When the spores thus produced are liberated as a result of the break-up of the sporophore, the empty shells become visible. The cell wall is soluble in 10 per cent KOH solution and in antiformin. When treated with concentrated H_2SO_4 it is first pigmented dark and is then dissolved.

As the great majority of the cultures do not show such septa, it has

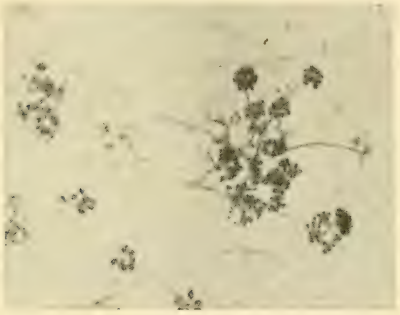

Fig. 12 b (*see* p. 52).

generally become recognized that an actinomyces colony represents a single-cell type. Drechsler (97) considered the actinomyces mycelium to be definitely septated, the hyphae being divided into short sections. This phenomenon is particularly striking in cultures belonging to the genus *Nocardia*, but appears only seldom among species of *Streptomyces*. Orskov (328) also described septa in certain cultures. He believed that this is the first stage in the process of the break-up of the mycelium into fragments. Krassilnikov (234) considered the observed formation of septa as merely the beginning of the fragmentation process.

In recent studies on the cytology of actinomycetes, using a more refined method of staining, namely, the tannic acid-crystal violet method, septa have been demonstrated (222) conclusively. They are formed early in the vegetative mycelium. This mycelium, however, although septated, never breaks up into single cells.

The mycelium of actinomycetes produces true branching of a monopodial type. Some observers have reported dichotomous branching (98), a phenomenon considered by others as uncertain (97).

The formation, by certain species, of nodes from which side branches are produced in the form of whirls has been reported by Waksman for *S. reticuli;* this was later confirmed by others, notably by Kriss (242), who added another species under the name of *S. verticillatus.*

The formation of short side branches which give rise to single spores is characteristic of species belonging to the genus *Micromonospora.* These spores have often been designated as chlamydospores or megaspores. Jensen (186) looked upon them, however, not as involution forms but merely as a type of development of rod-shaped cells, often observed among the mycobacteria and the corynebacteria.

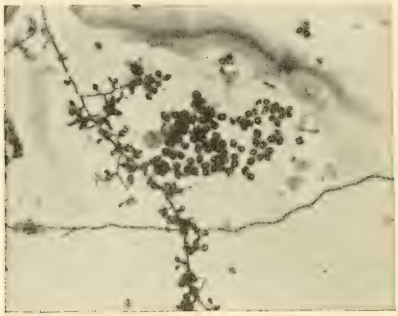

Fig. 12 c (*see* p. 52).

Among species of *Nocardia,* Lieske observed the production of swollen cells, which he considered as involution forms. Krassilnikov considered these as normal stages in the life cycle of the organisms.

In old cultures, certain swellings of the terminal ends of the hyphae may be observed. These may also be formed under abnormal growth conditions, as in concentrated media or in the presence of substances like caffeine. These swellings may be considered as involution forms, somewhat similar to the clubs produced by pathogenic actinomycetes in the animal body. The separation of actinomycetes on the basis of these formations is open to criticism.

Plasmolysis has not been established as yet for the actinomycetes with any degree of certainty. The lytic reactions are due either to autolytic enzymes or to specific phages.

Sporulation of Actinomycetes:—

Spore formation.—The actinomyces spore has been described as containing a spherical, relatively large chromatin body which is surrounded

by cytoplasm enclosed in a spore case. When the spore germinates, the chromatin bodies divide, some of the material entering the germ tubes. As the mycelium develops, it becomes filled with granular or rod-shaped chromatin bodies.

Lachner-Sandoval (247) was the first to recognize, in 1898, the true manner of sporulation among the actinomycetes. This was believed to be a distinguishing character of the organisms. Two types of spores were found to be produced, both asexually, one by the process of fragmentation and the other by the process of segmentation.

The fragmentation spores were looked upon as analogous to spores formed by true fungi. They are formed by the breaking up of the protoplasm within the cell wall into particles or fragments, more or less uniform in size. These fragments are later liberated by the splitting of the cell wall. During the contraction of the fragments, empty and clearer partitions are formed between them, which have been occa-

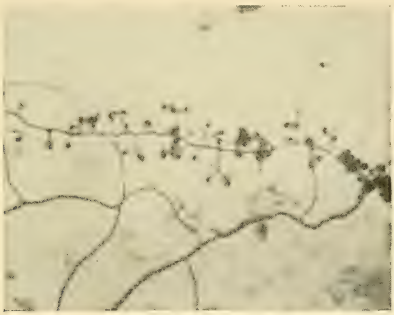

Fig. 12 d (*see* p. 52).

sionally taken for cross walls. When the spores mature, the surface cover becomes less defined and may gradually disappear, as a result of autolysis. The spore-bearing threads thus assume the appearance of chains of cocci, the spores falling apart readily. The surface cover may persist, however, without dissolving, in which case the spores leave through the broken ends of the sporulating hyphae. Sporulation by the fragmentation process begins at the top of the aerial hyphae and proceeds toward the base. This manner of sporulation is characteristic of the genus *Streptomyces*.

Sporulation by segmentation consists in the simple breaking up of the sporulating hyphae by means of cross walls. At first the hyphae are unicellular. At a certain stage of growth, cross walls are formed and the hyphae break up into small segments. These are cylindrical in form, with sharp edges and are uniform in size, usually 1-2.5 × 0.7-0.8μ. These often have been considered as true oidiospores (321).

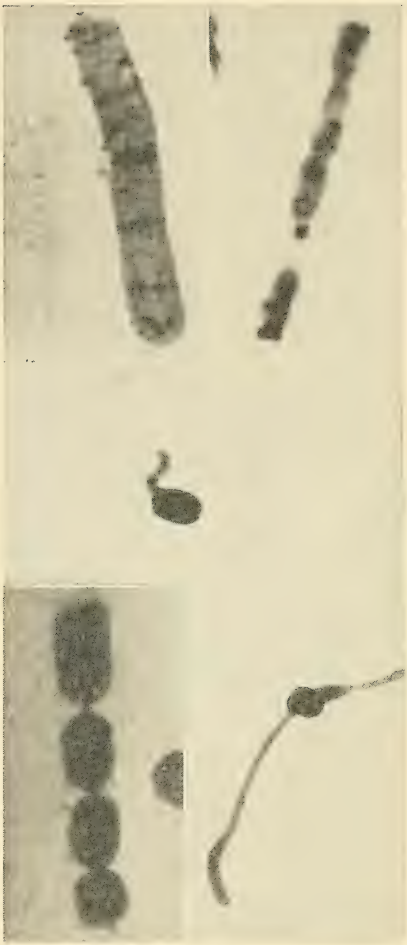

Fig. 13.—Details of sporulation and of spore germination by *S. griseus* as shown by electron microscope: Top, left, aerial sporogenous hypha showing septation prior to spore formation; top, right, more advanced stage in spore formation; bottom, left, well matured, four-spored chain; center, spore germinated by a single germ tube; bottom, right, spore germinated by two germ tubes (*from* Carvajal, 64). Note (top, left) mitotic division taking place in developing spores.

The cylindrical oidiospores may swell, giving rise to spherical bodies. This manner of sporulation is characteristic of the genus *Nocardia* and of certain species of *Streptomyces*.

Drechsler recognized three types of sporulation: (*a*) by means of true fragmentation, (*b*) by means of doubling of the cell wall, (*c*) by means of contractions similar to segmentation. According to Duché, the last process alone results in the production of three types of spores: (*a*) regular and irregular arthrospores, (*b*) microarthrospores, produced in the substrate mycelium, and (*c*) endospores in the aerial mycelium. The true conidia or fragmentation spores are formed only in the aerial mycelium, whereas the vegetative mycelium gives rise to chlamydospores or arthrospores.

These chlamydospores are produced by the concentration of the plasma in the substrate mycelium and are abundant in some species. They are spherical spores (1.5-1.7μ), with thick plasma, and are separated from the rest of the hyphae by cross walls. They are distinguished from involution forms by a thicker, light-reflecting plasma. They are not produced readily on protein media.

Spiral formation.—The sporophores in the aerial mycelium are either straight or spiral-forming. The manner of spiral formation is described in detail by Krassilnikov (236). The spirals in the mycelium curve not long before the spores are produced; the branch may be curved completely or only at the end. The number of turns varies in accordance with the length of the spiral. There may be as many as 15 or as few as 1 to 3 turns; usually there are 5 to 6. Some species are characterized by long spirals and others by short spirals, some by compact and others by extended spirals. The curvature of the branches may be clockwise (dextrorse) or counterclockwise (sinistrorse). Drechsler considered the manner of spiral curvature as characteristic of the species. Since certain species show both types of curvature in the same culture, this distinction can hardly be accepted.

Not all the aerial hyphae give rise to spores, some of the hyphae being sterile.

Nature of spores.—The spores of actinomycetes are spherical (0.8-0.3μ in diameter), oval, or cylindrical (0.8-1 × 0.7μ). The shape and size of the spores are characteristic of the species, with a certain degree of gradation and variation. Actinomyces spores are reproductive bodies, comparable to fungus spores, rather than resistant bodies like bacterial spores. Actinomyces spores are destroyed by heat at 60° to 65°C. for 10 to 15 minutes. It has been (225) reported that the spores are somewhat more resistant than the mycelium.

When brought into a favorable medium, the spores swell and give rise to one to four germ tubes. The different spores vary greatly in this respect. Both the conidia and the oidiospores germinate in a manner similar to that of the corresponding spores of fungi. The germ tubes may appear at one end or at both ends of the cylindrical oidiospore.

Reproduction can also occur by the vegetative process, namely, through the growth of pieces of mycelium, and by the formation of buds, which gradually grow into branches, as well as by means of the chlamydospores. The germination of these spores is similar to that of the other reproductive bodies, independent of the hyphae in which they are produced (234).

FIG. 14.—Aerial mycelium of a *Streptomyces*, showing zonation or "fairy ring" formation (*from* LIESKE, 260).

Sporulation of the *Micromonospora* is distinct from that of the other genera. The monopodially branched mycelium is similar to that of the other actinomycetes. The conidia are formed on special branches, which are straight and short—5-10μ long—and which frequently give rise to other branches, thus producing group-like structures similar to bunches of grapes. Each branch bears at the end a single spore, pro-

duced by the splitting off of the tip of the hypha. The conidia are spherical (1.0-1.3μ in diameter), oval, or oblong (1.3-1.5 × 1.2μ). Sporulation occurs most abundantly on synthetic media.

Types of Growth on Solid and Liquid Media:—

Aerobic organisms.—The colonies of the aerobic actinomycetes, considered by some as pseudo-colonies, differ greatly from the colonies of fungi, on the one hand, and of the bacteria, on the other. They are usually compact, leathery, growing deep into the medium. Only certain few aerobic pathogens produce colonies of a dough-like consistency, which makes them similar to the colonies of bacteria.

The colonies can be round and smooth, or much-folded, lichnoid to almost barnacle-like in appearance. The edge of the colony, when examined under the microscope, gives a characteristic picture of radiating hyphae. The colony may be produced below or on the surface of the medium. In liquid media, the colonies may be formed individually on the bottom of the container, they may adhere to the surface of the wall of the container, or they may give rise to a ring of growth on the surface of the medium. The surface colonies may coalesce, producing a pellicle of varying degrees of compactness. The colonies may also be flaky in appearance, but they cause no turbidity of the medium. Sometimes the surface growth is similar to that of tubercle bacteria, that is, dough-like and folded, without producing any aerial mycelium. The composition of the medium has an influence upon the nature of the growth.

When actinomycetes are grown in a submerged or in a shaken condition (452, 517) they produce characteristic small, bead-like colonies, or granules which may completely fill the culture vessel. Probably because of the continuous break-up of the mycelium or the separation of the spores, growth is much more rapid and more abundant in submerged culture than in stationary culture. This is particularly true of certain species of *Micromonospora.*

Anaerobic organisms.—The morphology of the anaerobic forms represents a special problem. Erikson (112) made a detailed study of the morphology of 15 strains of the microaerophilic types of *A. bovis* derived from human materials and of 5 strains of bovine origin.

A very sparse development of erect aerial hyphae was detected when the human strains were grown in an atmosphere of reduced oxygen tension. These hyphae were found to be occasionally septate, but no definite spores were produced; they were of the same diameter as the hyphae of the substratum mycelium. The substratum mycelium is initially unicellular, the branches extending into long filaments, causing the colony to adhere to the medium. This mycelium may give rise to irregular segments, with a characteristic angular branching. The colonies were said to exhibit polymorphism, although no stable variants could be demonstrated. They gave no turbidity in the medium.

The colonies from the bovine strains were smoother and softer in consistency and did not adhere to the medium. Growth was scantier. The mycelium underwent fragmentation very rapidly, giving only traces of extensive ramification. No aerial hyphae were produced. In contrast to the human strains, the bovine strains showed occasional turbidity in the medium, and they were less able to ferment sugars, especially salicin and mannitol.

No filterable stage could be demonstrated by ultrafiltration experiments on either the human or bovine strains, and no evidence could be obtained in favor of any hypothetical life-cycle.

Autolysis of actinomycetes.—Only a few actinomycetes are able to show the phenomenon of autolysis. This was reported first for animal pathogens (91) and later also for plant pathogens and for soil saprophytes (509). The active agent responsible for the lysis was considered to be either an enzyme or a nontransferrable phage.

Of 1,000 or more freshly isolated cultures of actinomycetes studied by Krassilnikov (232) only very few were able to undergo lysis. An organism described as *A. albicans* at first gave a typical heavy compact growth covered with white aerial mycelium. On continuous transfer, the colonies became flat, smooth, and somewhat moist and lost the property of producing aerial mycelium. The culture was gradually reduced to a very thin slimy film. It grew more and more poorly, becoming, on repeated transfer, more rapidly transparent, until it finally ceased to grow altogether. All attempts to keep it alive were unsuccessful. Six other strains of a similar nature were isolated later. They belonged to different groups and showed different degrees of lysis.

Among actinomycetes, autolysis may not appear all through the colony, but may affect only certain sectors or spots, the unlysed part of the colony being quite distinct from the lysed part. Frequently lysis begins in the center of the colony and proceeds to the periphery.

The mechanism of autolysis among pathogenic forms is similar to that of the saprophytes but proceeds more rapidly (232). Meat-peptone agar is a favorable medium for the study of autolysis. When the culture is grown at 25°C., plated out and incubated at 30° or 37° (for pathogens), autolysis proceeds very rapidly; in fact, it becomes evident in 4 to 6 hours. Not all the hyphae are lysed uniformly. Some produce chlamydospores, and others, spherical bodies, as well as hyphal fragments. Under favorable conditions, all three types of bodies are able to grow and develop into fresh colonies.

The lytic factor is present within the cells of the actinomycetes. It becomes active when growth ceases, although it is possible that there is considerable overlapping of the two processes. When a growing culture is treated by physical or chemical agencies so as to stop growth, lysis begins immediately. If the temperature of the culture is raised to 60°—70°C., autolysis occurs in a few minutes. The lytic factor is in-

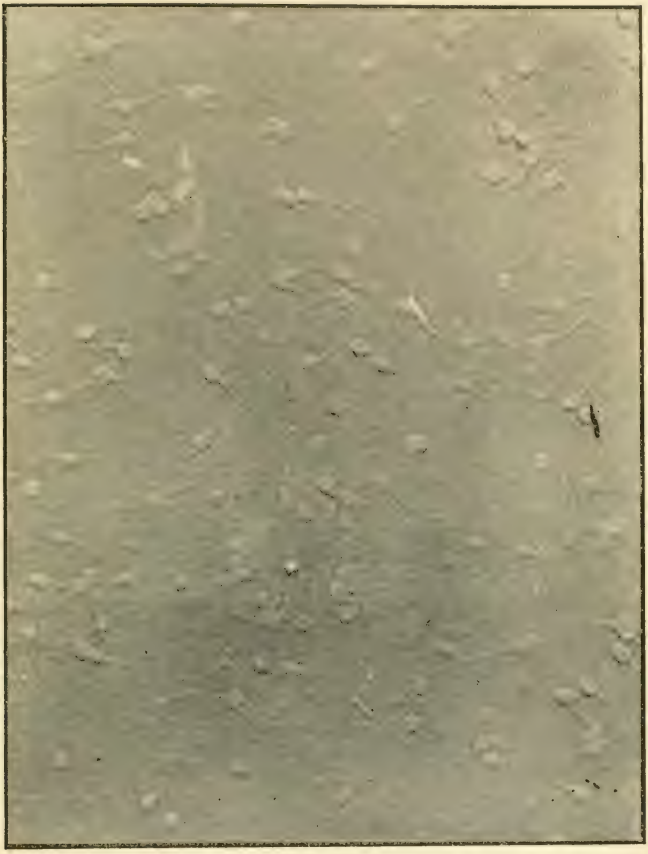

Fig. 15.—Electron micrograph of actinophage (*from* Woodruff, *et al.*, 518).

activated when the culture is heated to 100°, but not to 80°, for 5 minutes.

The lytic factor of actinomycetes is very specific. It does not act upon other species or even on closely related forms. It is not to be confused with the transmissible or phage factor which affects certain actinomycetes. In contrast to actinophage, the lytic agent acts also upon the dead cells of the organism.

A thermophilic actinomyces isolated (207) from composts of horse manure was found to grow well on various media, but it underwent lysis when grown in a synthetic medium containing ammonium sulfate and starch, after 24 to 48 hours' incubation at 50°C. During growth, the *p*H of the culture changed from 7.0 to 5.7. The addition of $CaCO_3$ to the medium prevented the production of acid as well as of lysis.

After maximum growth has been attained, *S. griseus,* the organism that produces streptomycin, undergoes lysis (395). This takes place more rapidly under submerged than under stationary conditions of cultivation. The whole culture tends to become viscous as a result of formation of the lysed material. Apparently the maximum peak of streptomycin production is associated with the setting in of lysis of the culture. When lysis has progressed too far, production of streptomycin ceases, and even that already produced may be destroyed.

Dmitrieff and Souteeff (91) observed that a culture of an organism designated as *Actinomyces bovis,* and which evidently belonged to the genus *Streptomyces,* underwent lysis in various media. When the organism was grown on agar media, the production of lysis was found to be associated only with the formation of a certain type of colony. As a result of lysis, two types of daughter colonies were formed: one was similar to the mother colony and was characterized by capacity for lysis; the other type of colony did not lyse and was morphologically different from the first. The cultures that originated from the colonies capable of undergoing lysis were strongly proteolytic and did not form any aerial mycelium. The cultures obtained from nonlysing colonies were less proteolytic and produced a chalky aerial mycelium, which changed the reaction of litmus milk to alkaline. In broth cultures, lysis took place in 2 to 3 weeks; it was associated with the living organism and was of the nature of a nonenzymatic and nontransmissible lytic factor.

These results are comparable to those obtained later by Schatz and Waksman (395) in the production of inactive strains by *S. griseus.* These strains were free of aerial mycelium, produced no streptomycin, underwent much more rapid lysis, and formed much more acid in the medium (Table 5).

Effect of actinophage upon actinomycetes.—Wiebols and Wieringa (509) observed that cultures of actinomycetes isolated from infected potatoes underwent lysis. This phenomenon was ascribed to the

production of specific transmissible phages. Repeated additions of a filtrate of *S. roseus* to the culture of the organism resulted in the development of a phage which gave a large number of plaques on solid media inoculated with the actinomyces and inhibited the growth of the organism in liquid media. Phages were also obtained from the patho-

TABLE 5: *Cultural and physiological characteristics of the streptomycin-producing strain of S. griseus and its inactive variant* (395):—

ACTIVE STRAIN	INACTIVE VARIANT
1. *Antibiotic activity.* Produces streptomycin in both shaken and stationary cultures.	1. No streptomycin formed either in shaken or stationary cultures.
2. *Growth.* Surface growth always heavily sporulated; grayish-green aerial mycelium.	2. No sporulating aerial mycelium; scant development of aerial hyphae with slight tendency to form spores in some old cultures.
3. *Reaction.* Medium always changes to alkaline; *p*H 7.5–8.5.	3. Reaction of medium at first acid, *p*H 5.0–6.5; later becoming alkaline.
4. *Glucose.* Glucose completely consumed in 6–8 days in stationary cultures and in 3–4 days in shaken cultures.	4. Glucose utilized more slowly.
5. *Lysis in shaken cultures.* Shaken cultures produce very fine flocculant growth, tending to lyse slowly after about 15 days.	5. Cultures produce at first balls of growth which change into the turbid, flocculant type; rapid and complete lysis in 7–10 days.
6. *Lysis in stationary cultures.* Surface pellicles stable; any submerged, flocculant growth tends to lyse as the surface pellicle develops.	6. Stationary cultures produce no surface growth but flocculant, submerged mycelial growth which lyses slowly, only after a month or longer.
7. *Viscosity.* Culture filtrate not showing any viscosity.	7. Culture filtrate becomes viscous during or after lysis.
8. *Reinoculation.* Inoculation of cultures with lysed inactive culture induces no lysis or reduction in activity.	8. Inoculation of cultures with spores of active strain produces growth and antibiotic activity if some glucose remains.
9. *Variation.* Sporulating strain gives rise to non-sporulating variants.	9. Asporogenous variants may reconvert to active, sporogenous forms.
10. *Sensitivity to streptomycin.* Very resistant to this antibiotic.	10. Very sensitive to this antibiotic.

genic organisms *A. bovis* and *N. farcinica.* A polyvalent phage was obtained from one of the actinomycetes which was also active upon *S. scabies,* thus suggesting possible methods of combating potato scab. The phenomena of phage production by actinomycetes was referred to as "microbiophagy." These investigators were thus the first to emphasize the existence of filterable and transmissible agents comparable to bacteriophages, which were active upon actinomycetes.

KRASSILNIKOV and KORENIAKO (237) emphasized the resemblance of the process of autolysis among actinomycetes to the action of phage

upon bacteria. They reported, however, that the lytic factor of actinomycetes was highly specific, since it had no effect upon other species or even upon other strains of the same species of actinomyces. When growth of the organism was delayed under the influence of various factors or when the culture became aged, lysis took place. Different cultures underwent lysis with varying degrees of rapidity. It was assumed, therefore, that production of the lytic factor or its mode of action differed with the various organisms. At temperatures of 60° to 70°C., lysis occurred in a few minutes. The lytic agent was resistant to a

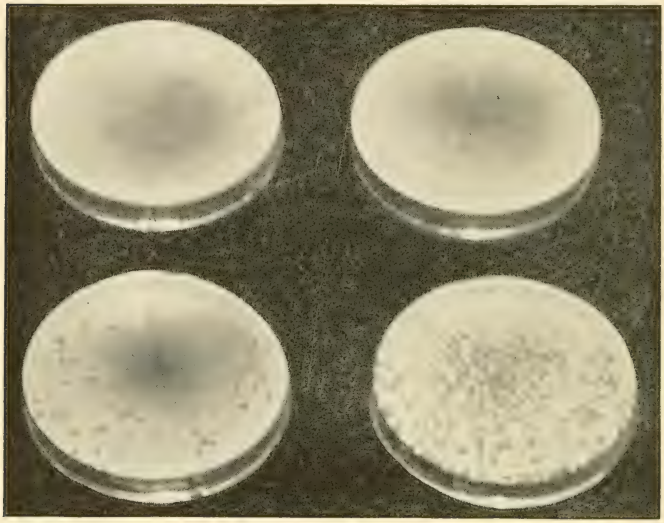

Fig. 16.—Method of measuring actinophage concentration (*from* Reilly, Harris and Waksman, 355).

temperature of 80°C. for 1 hour but was destroyed at 100°C. in 5 minutes. Not only the living but also the dead cells of the organism were affected by the lytic agent. This last suggested that the agent is different in its action from that of true phage.

With the discovery that the streptomycin-producing strains of *S. griseus* are subject to attack by a virus or a phage-like agent, the problem of phage action upon actinomycetes entered a new phase. The lysis of the actinomyces produced by the phage appeared to be quite distinct from the lytic phenomena.

Saudek and Colingsworth (383) were the first to report that *S. griseus* is subject to the action of a transmissible lytic agent which had

all the properties of phage. In the presence of young cultures of S. *griseus*, the phage developed rapidly and brought about the lysis of the culture. The plaque method was used for measuring the concentration of the phage. Streptomycin production was partly or completely prevented by the phage. Cultures of S. *griseus* resistant to the action of the phage could easily be isolated.

Woodruff and Foster (516) exposed to laboratory air for 24 hours a submerged culture of S. *griseus* in a stationary condition, with plugs removed from the flask. The freshly formed pellicle showed evidence of plaque formation. The same phenomenon was observed in a factory 500 miles away. Upon transfer of a filtered culture into a fresh culture of S. *griseus*, the phage multiplied. It was calculated that after six transfers, each phage particle increased to 75×10^{20} particles. The phage was active against all streptomycin-producing strains of S. *griseus* but not against the non-streptomycin-producing strains. Phage-resistant strains developed readily. They retained their capacity to produce streptomycin but were not absolutely free from phage. The phage of S. *griseus* had properties similar to those of bacterial phages, as shown both by cultural characteristics and by appearance in photographs made by means of an electron microscope (Fig. 15).

The following method can be employed (355) for assaying the activity concentration of phage in a given preparation. A 3 to 5-day-old shaken culture of a streptomycin-producing strain of S. *griseus* is filtered aseptically through paper and inoculated on plates. The phage preparation is obtained by inoculating with phage, young cultures of S. *griseus* grown in a shaken condition, allowing the cultures to incubate further for 24 to 72 hours, and filtering them through a Seitz filter. Dilutions of phage, ranging from $1:10^6$ to $1:10^{12}$, are added to 10-ml. portions of sterile nutrient agar, previously inoculated with 0.1-ml. portions of the paper-filtered culture. The agar is poured into plates, which are incubated at 28°C. for 2 days. The plaque counts are made, as shown in Fig. 16, and calculated for 1 ml. of the preparation. Some preparations gave 4×10^{10} or more particles per milliliter.

The phage preparation is kept in the refrigerator and used as a standard. To illustrate the effect of actinomyces inoculum upon the phage count, three different concentrations of filtered 7-day-old shaken culture of streptomycin-producing S. *griseus* were added to nutrient agar. The plates were inoculated with the same amount of the phage and incubated at 28°C. for 48 hours. The following results were obtained.

Inoculum per cent	Plaque counts $\times 10^7$
10.0	391
1.0	698
0.1	756

These results show that the plates do not have to be heavily inoculated with S. *griseus* in order to give uniform growth on the plate of the organism subject to attack by phage, with the resultant formation of plaques.

Actinophage of S. *griseus* was found to attack only the streptomycin-producing strains of this organism. It had no effect on other strains of S. *griseus* or on other streptomycin-producing organisms such as S.

TABLE 6: *Effect of phage upon the growth, phage multiplication, and streptomycin production by different actinomycetes in stationary cultures* (355):—

		9 days		13 days	
ORGANISM	Phage added*	Phage per ml $\times 10^7$	Streptomycin	Phage per ml $\times 10^7$	Streptomycin
Streptomycin-producing strains of *S. griseus*			gm/ml		gm/ml
No. 3463	0	–	–	0	21
	+	–	–	200	5
No. 3475	0	0	30	0	180
	+	>50	<5	370	<5
No. 3480	0	0	31	0	189
	+	10	<5	30	28
No. 3481	0	0	73	0	174
	+	50	<5	260	13
No. 4	0	0	43	0	201
	+	30	<5	160	<5
3475-2PR	0	>0.01	40	40	129
	+	>50	16	370	75
Grisein-producing strain of *S. griseus* 3478	0	0	<5	0	<5
	+	0	<5	0	<5
Inactive strain of *S. griseus* 3326a	0	–	–	0	<5
	+	–	–	<0.2	<5
Streptomycin-producing *S. bikiniensis*	0	0	<5	0	30
	+	3	30	7	33

* Each 60-ml flask of culture received at start 0.1 ml of M-1 phage, amounting to 7×10^7 particles per 1 ml of medium.

bikiniensis. In cultures that do not produce streptomycin, the phage did not multiply and in some cases was destroyed or absorbed (TABLE 6). This actinophage multiplies only at the expense of the living cultures of S. *griseus* but not on the heat-killed organism. Its optimum temperature for multiplication is 28°C., and it does not grow at 37°C. or above. However, it can withstand a temperature of 75°C. for 1 hour but is completely destroyed when heated at 100°C. in 10 minutes. When it is stored at 6°C., there is little loss of activity, but storage at

28°C. or at higher temperatures results in loss of activity, the rate of loss being proportional to the temperature (Table 7).

Constancy of actinomyces types.—Each one of the four genera of actinomycetes has clearly defined morphological characters. Although there is a certain amount of overlapping between the species within the different genera, notably between the species of *Actinomyces* and of *Nocardia,* or between *Nocardia* and *Streptomyces,* the combined morphological and cultural properties well characterize each genus. Very often a species of *Streptomyces* may lose, by selection or by mutation or natural variation, the property of forming aerial mycelium; it may then appear to become a typical *Nocardia.* This was shown to hold true, for example, of the streptomycin-producing strain of S. *griseus.* When such a change occurs, it is accompanied by a change in the physiology of the organism. Usually, however, the culture reverts to its original form under proper methods of cultivation.

Table 7: *Stability of phage in aqueous suspension upon storage at several temperatures* (355):—

Temperature of storage,	Phage particles $\times 10^7$ per ml, after storage*		
	3 days	12 days	29 days
°C.			
6	44	–	60
28	31	20	0.00005
37	37	15	0.0000009
56.5	18	0.001	0

* At start all preparations contained 36×10^7 particles of phage per ml.

This emphasizes the fact that there is a marked interrelation between the morphological and physiological properties of an organism. Ample evidence of this has been established for the rough and the smooth strains of bacteria. Apparently such interdependence, though of a somewhat different kind, exists also among actinomycetes.

The four genera of the actinomycetes have been shown to possess constant morphological properties, with a limited overlapping of the different genera. These properties may be summarized as follows:

The genus *Actinomyces* comprises the anaerobic pathogenic forms. It is characterized by a gram-positive, non-acid-fast, branching vegetative mycelium. No aerial hyphae are formed. The mycelium tends to break up into bacillary forms.

The genus *Nocardia* is characterized by the formation of an undivided substrate mycelium in the early stages of development. Aerial mycelium may be formed among certain members of the group, but it is usually indistinguishable from the substrate mycelium. The nonseptated hyphae of both the substrate and the aerial mycelium break

apart into short rods and cocci, by a process of segmentation, comparable to oidia formation. The spores germinate, giving rise to a true mycelium. Some of the members of this group are characterized by a marked plemorphism, being either acid-fast or non-acid-fast. The angular type of growth described for some of the actinomycetes is also a property of certain members of this group. In recent studies on the nocardias, Erikson (115*b*) examined 300 strains, freshly isolated from soil or obtained from culture collections. On immediate isolation, only 9 per cent were partly acid-fast, but on subsequent cultivation on organic matter-rich media, this increased to 31 per cent. These strains ranged from those giving soft mycobacterial type of growth with transient vegetative mycelium and very sparse aerial mycelium to the harder streptomyces-like varieties. No evidence was obtained of any resting spores or chlamydospores in the vegetative mycelium; the aerial mycelium, if present, does not form any true spores. The nocardias were, therefore, considered as asporogenous.

The genus *Streptomyces* produces a well-developed nonseptated mycelium. The vegetative mycelium does not divide during its development but gives rise to a somewhat thicker aerial mycelium, which is formed most readily on synthetic or poor media. The aerial hyphae produce straight or curved sporulating branches. These give rise to conidia, by a process frequently designated as fragmentation. The spores are produced within the sporulating hyphae and are separated from one another by a constriction process. Later they are liberated by constriction of the cell wall and, its subsequent dissolution. The process of segmentation or oidia formation may also occur among the members of this genus. The substrate mycelium may produce chlamydospores; the broken bits of mycelium also have the capacity of growing into a fresh mycelium.

The genus *Micromonospora* is characterized by the formation of a well-developed branching mycelium, producing single oval spores on the tip of special sporophores or side branches. These spore-bearing branches may be single or much-branched, the latter giving rise to a mass of spores similar to a bunch of grapes. No surface growth is produced in liquid media, but abundant growth is formed when such media are stirred or shaken at frequent intervals, thus breaking up the spores, which give rise to new clumps or colonies within the media.

Micromonospora may be looked upon as the most highly developed group among the actinomycetes, placing the whole order Actinomycetales closest to the fungi. On the other hand, the genus *Nocardia* is in many respects related to the mycobacteria, and, through them, to the true bacteria.

Chapter IV

VARIATIONS AND MUTATIONS

No other branch of biology offers so rich a field for the study of variations and mutations and for the rapid selection of new varieties, as that of microbiology. This is due to the simple fact that many generations of organisms can be obtained in a very short time. The fact that these organisms can be grown in an absolutely pure culture, free from any other organisms, and that the composition of the medium and the conditions of growth are easily controlled are other contributing factors. Among the various groups of microorganisms that are readily subject to variations and mutations, the actinomycetes occupy a prominent place.

Actinomycetes are greatly influenced, especially in their cultural characteristics, by the composition of the medium and by the conditions of growth. Variations resulting from cultural differences have often led to expressions of doubt concerning the existence of definite types or species among the actinomycetes (261). Because of this doubt, the use of "species-groups" rather than of definite species for the classification of actinomycetes has been suggested (443).

The general appearance of the actinomyces colony, the abundance and formation of aerial mycelium, the manner of sporulation, the production and nature of endopigments and exopigments, and the vitality of the organism when grown on different media make up the variation complex of actinomycetes.

Types of Variation:—

General variations.—Early students of actinomycetes recognized the fact that variations among actinomycetes are of several types. Lieske (260) demonstrated that actinomycetes show greater variability in their morphological and physiological properties than do any other group of microorganisms. He classified the types of variations as (*a*) simple modifications, (*b*) permanent modifications, and (*c*) mutations, including the formation of sectors within a colony. Waksman (446) emphasized that the variations among actinomycetes differ in quantity and in quality, not only under the influence of various environmental conditions but even on continued cultivation under the same conditions. The soluble pigment may be lost or changed in color; the color of the aerial mycelium may change; even the property of forming aerial

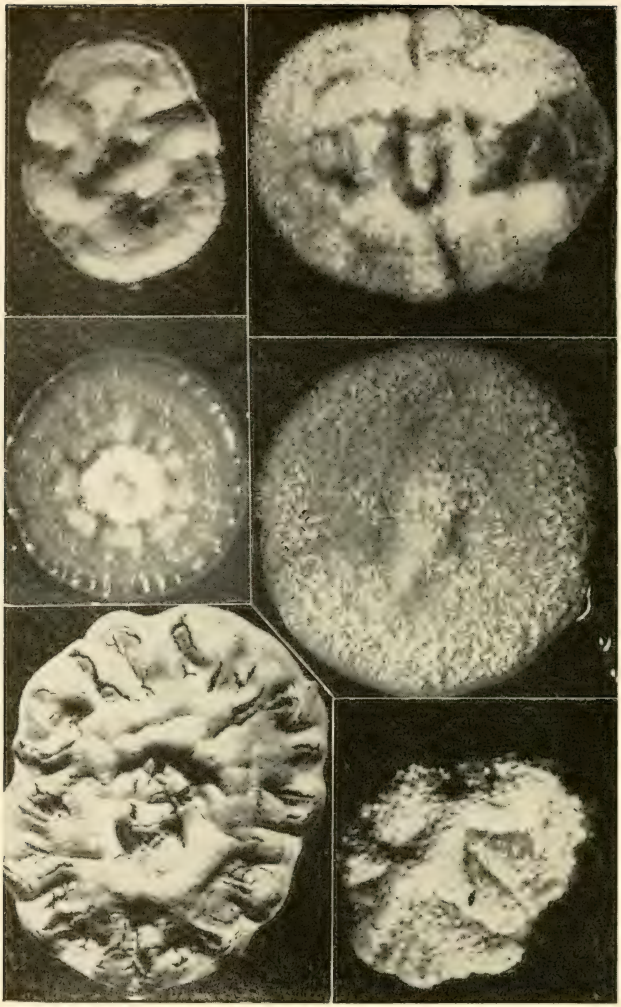

FIG. 17.—Variants of *Streptomyces griseus* growing on yeast extract glucose agar (*from* DULANEY, RUGER, HLAVAC, 101a).

mycelium may be lost. The size, shape, and color of colonies, the length and abundance of mycelium, and the manner of spore formation are influenced by the composition of the medium and the age of the culture.

In more recent studies (199) the general variations among the actinomycetes have been divided into three classes: (*a*) adaptive, or amenable to environment; (*b*) continuous or fluctuating, as shown by differences in the colonies plated out from the same culture; (*c*) developmental, resulting in saltations or mutations. The adaptive type is usually characterized by a decrease in the size of the colony, a loss of the capacity to form sporogenous hyphae, a reduction of the ability to utilize certain nutrients, a change in pigment production, and a loss in the capacity to produce antibiotic substances. The continuous type is most clearly marked by the nature and intensity of the pigment produced by the organism, as well as by the capacity to produce a given antibiotic. The developmental variations are expressed in the presence or absence of aerial mycelium, pigmentation of the vegetative or aerial mycelium, and production of antibiotics. Some of these changes can be reversed to the original by growing the organism on special media, such as glycerol nutrient agar, or in some natural medium, such as sterile soil. Other variations or mutations are more permanent or stable in nature, although only on rare occasions.

In spite of these many types of variations, the constancy of strains or species of actinomycetes can be maintained if proper care is taken in growing the cultures on suitable media. The recognition of this fact has led some investigators (98, 318) to emphasize the constancy of the characters of actinomycetes, as contrasted to others who denied such constancy.

Tempel (414) observed that several actinomyces strains failed to show, under constant conditions of culture, any sudden changes either in morphology or in physiology, which could be considered as mutations. The physiological changes due to the effects of temperature, aeration, reaction and composition of medium were confirmed, but these changes were not permanent in nature. Rippel and Witter (361) could not obtain any variability among several actinomycetes, either by changing cultural conditions or by irradiation by means of Röntgen rays or ultraviolet rays. Spontaneously occurring sectors gave normal cultures on transfer.

Hereditary variations.—Several specific forms of hereditary variation among actinomycetes have received particular consideration. It is sufficient to mention the following: (*a*) transformation of an actinomyces into a mycobacterium-like organism (328, 378), the former being regenerated by cultivation on certain media, such as potato; (*b*) transformation of an actinomyces into diphtheroid organisms (211); (*c*) transformation of anaerobic, short hyphal-producing forms into aerobic, long hyphal forms (328); (*d*) change of aerial mycelium and strepto-

mycin-producing strains of *S. griseus* into inactive strains free from aerial mycelium (395); (*e*) change of strain of *S. griseus* from a colorless vegetative culture to a pink variant, accompanied by a change in antibiotic-producing capacity.

Early students of the actinomycetes (388) observed that the acid-fast organism which causes infection in man gives rise to two subtypes, one simple in nature and liquefying gelatin, and the other producing pseudotubercles and not liquefying gelatin. The second form was looked upon as intermediary between actinomycosis and tuberculosis.

Numerous references are found in the literature (188) to the transformation of actinomycetes, under special conditions of culture, into mycobacterium-like organisms, or into diphtheroid organisms, and *vice versa*. Of special interest are the transformations of anaerobic, short-hyphaed forms of actinomycetes into aerobic, long-hyphaed forms. Dissociation of pathogenic actinomycetes into aerobic and anaerobic strains has frequently been reported (427). It has also been reported (319) that two sorts of anaerobic colonies were isolated from the pus of actinomycosis, one smooth and composed of gram-negative rods, and the other adherent and composed of gram-positive filaments. These were looked upon as S and R forms of the organism; even a transitional O form was recognized. These variations have often been considered as a part of the life cycle of the organisms. The composition of the medium, that is, whether complex organic or simple inorganic, protein-rich or carbohydrate-rich, and its reaction greatly influence the stability of the culture, or the cycle of growth of actinomycetes. This is true also of environmental factors, especially moisture content, aeration, and temperature. The presence of other organisms, resulting in antagonistic and associative effects, likewise influences the variation of the culture.

Individual variations and group variations may also be distinguished. The size of mycelium fragments, the formation of grains in the disintegration of the cells, the formation of conidia and chlamydospores—all influence the cycle of growth of the individual organism, with the resulting variations and modifications.

The problem of cell polymorphism among actinomycetes has also aroused much attention. This property must be taken into consideration in placing any organism in its taxonomic position (191). The formation of new races or strains can be accounted for on the basis of changes, which are expressed by the surface appearance of the colony, whether smooth or rough, by the presence or absence of aerial mycelium, by the manner of sporulation, by changes in pigmentation, and by other cultural characteristics.

Mutations.—The formation of saltants or mutants by actinomycetes must be regarded as in a class by itself, distinct from the variants. The mutations may be said to include the following types: formation of white strains from blue forms; formation of strains free from aerial mycelium from strains producing such mycelium or *vice versa;* formation

of sectors pigmented red among orange-yellow strains. These saltations, are accompanied by morphological, cultural, and physiological characters which are quite different from those of the mother cultures. These new strains are so distinct that they might be considered new species, in accordance with the accepted systems of classification.

Stable mutants or saltants were obtained and studied in detail by Kriss (242) and Krassilnikov (234). Jensen has shown (188) that under the influence of ultraviolet rays or even spontaneously, two strains of *Nocardia* isolated from Australian soils gave rise to new forms, some of which resembled typical species of *Streptomyces* and others of which were closer to the mycobacteria. Jensen (191) also observed that under the influence of LiCl mycobacteria gave rise to forms that might be considered as species of *Nocardia.*

Recently, extensive investigations have been made of the effect of ultraviolet radiations and x-rays in inducing mutations of various species of *Streptomyces.* Savage (385) reported that ultraviolet rays were less mutagenic than the x-rays, the harder rays of 0.710A and 0.210A wave lengths being most efficient. Mutation rates increased with killing rates up to 99.9 per cent of killing. When doses of 1,000,000 roentgens were used, as high as 50 per cent mutation rates were observed on morphological properties and 40 per cent on streptomycin production.

By means of x-ray and ultraviolet light irradiations, Kellner (213) found that most antibiotically inactive cultures gave rise to antibiotic-producing mutants. Of the greatest interest was the fact that a strain of S. *griseus* kept for a long time in the culture collection and which was inactive antibiotically was induced to form a mutant which produced streptomycin. The frequency of active mutants ranged from 0.01 to 1.2 per cent; mutants obtained from the same parent culture varied in their antibiotic spectra. The viability of conidia exposed to ultraviolet irradiation could be recovered by illumination with visible light (214).

The varations or mutations may thus influence not only the species characteristics but also the generic characters. Krassilnikov emphasized that these changes take place from the simpler to the more complex forms, as from micrococci to mycobacteria, from mycobacteria to nocardias, and from nocardias to streptomyces; the reverse phenomenon occurs but seldom. This reasoning led Krassilnikov to the conclusion that actinomycetes are present in natural substrates, such as soil, largely in the form of micrococcus stages.

Kriss (240, 242) recognized four types of variation—morphological, cultural, physiological, and applied. These may be briefly summarized as follows:

Morphological variations.—Some of the morphological variations reported may be considered here in further detail. Jensen (188) described the production from single-cell cultures of *Nocardia polychromogenes* of two different forms, one a rod-shaped or R-form, and the other a filamentous or F-form. The R-form produces initially a

small unicellular mycelium which soon divides into bacteria-like elements; these multiply by cell division in the manner characteristic of corynebacteria. Two subtypes were recognized for the R-form: the soft or s-type and the hard or h-type. The s-type, which is the original, produces a soft, pasty growth of red color; the bacteria-like elements are usually short, blunt, little-branched, and partly acid-fast. The h-type produces a dry, crumbly growth, adhering firmly to the medium and consisting of longer and more slender cells, less acid-fast than the s-type and with a marked tendency to form long filaments. The h-type arises spontaneously in, and can also be produced experimentally from, cultures of the s-type. Exposure of the h-type to ultraviolet rays gave rise, for example, to a yellow and a white variety of the s-type. The s- and h-types were believed to correspond to the plane and perrugose variants of mycobacteria, and were also comparable to the smooth and rough variants among other bacteria. The F-form represents a stabilization of the initial mycelial stage of the R-form. It is an actinomyces-like organism, consisting of long, delicate, branching hyphae, with a well-developed aerial mycelium, and without any tendency to divide by septa into bacteria-like elements. The F-form was found to arise spontaneously in old cultures of the s-type, but not in the h-type. Its appearance did not seem to be influenced by external factors.

Novak and Henrici (326) reported the appearance of a yellow staphylococcus in a Berkefeld filtrate of a broth culture of a saprophytic actinomyces. Under the microscope, the staphylococcus was observed to change first into rods, then into long, branched filaments which could not be distinguished from true actinomyces mycelium. The reverse changes were also observed. The coccus was found to dissociate first into S- and R-forms, then into filterable G-forms. These observations were believed to support the theory that staphylococci are related to the actinomycetes. As pointed out above Krassilnikov described the micrococcus as merely a stage in the normal development of the nocardia rather than as an abnormal mutant.

Certain of the characters of actinomycetes appear, however, to be far more constant than those listed above. These include the formation of aerial mycelium on specific media, the formation, nature, and direction of the spirals, the manner of spore formation, and the size and shape of spores. Only seldom do variations occur in such specific characteristics as abundance of the mycelium, lengthening or shortening of hyphae, and size of spores (242).

Cultural variations.—Among the cultural variations, those of pigmentation are most striking, since pigments are widely distributed among actinomycetes. This is of particular significance in view of the fact that differentiation of many species is based upon the nature and intensity of the pigment. Even the major subdivisions of some of the groups of actinomycetes have been based upon pigmentation, as was done by Sanfelice, Duché, and others. Evidence of this is found in

the designation of such groups as *albus, flavus,* and *violaceus.* Waksman also proposed a key for the separation of species of actinomycetes on the basis of the pigment produced on organic and synthetic media, including soluble and insoluble (or exo- and endo-) pigments.

More detailed study has revealed, however, that on continued cultivation of organisms, the pigment undergoes changes in its nature, or it disappears altogether. Thus an organism designated as *A. verne,* because of the soluble green pigment produced in the medium, lost that property on continued cultivation. When the characters of an organism are based on pigmentation, it becomes very difficult to make comparisons even if type cultures are available. Thus, one of the most widely used cultures of actinomycetes, the streptomycin-producing strain of *S. griseus,* can hardly be recognized either by comparison with the original cultures of Waksman and Curtis or from the original description of Krainsky, since the type culture lost its characteristic pigmentation and Krainsky's description did not quite correspond with the published description of its pigmentation.

Among the other cultural variations reported for actinomycetes, the lytic activities of many of the strains deserve consideration, as pointed out previously (p. 60). The phenomenon of lysis, whether considered as a part of the life cycle of the organisms or looked upon as stages of degeneration of a culture, has a bearing upon the production of new types. This holds true also for the effect of phage upon the development of resistant strains.

Marked variations in agar-decomposition and pigmentation of *S. coelicolor* have also been observed (408). Erikson (115*a*) found that the major variations of *S. coelicolor* comprise loss of pigmentation, loss of aerial mycelium, and occasionally also loss of agar-liquefaction. Single spore isolations from aerial mycelium brought out the possibility of inherent differences in the sister spores of the same chain. Spontaneous occurrence of variants may be found more readily in the spores of degenerate colonies, rendered atypical by artificial methods of cultivation, than in the spores of the aerial mycelium of typical colonies. In an agar-liquefying strain, 3 out of 15 spores lost the power to produce the pigment and to liquefy agar. A non-agar-liquefying strain, which had lost the power of pigmentation, gave a variant which produced sectored colonies, some of which possessed the blue pigment.

Physiological and applied variations.—These can best be described by an analysis of the variation of two important economic groups of actinomycetes, namely, those that cause potato scab and those that produce antibiotics. These physiological variations are usually more quantitative than qualitative in nature.

Potatoes show considerable variation in their resistance to scab. This has been ascribed either to differences in the environment in which the potatoes are growing or to physiological differences of the strains of *S. scabies,* the causative agent of infection.

Schaal (387) has recently shown by means of sectoring of S. *scabies* strains that as many as nine sectors appeared in a single colony. The sectors varied in the nature of their mycelium, in the rate of growth of the culture, and in pigmentation. Thus the variants showed not only differences in physiological characteristics from that of the parent culture, but even in morphology. The formation of spirals and the direction of turns varied with the culture. There was little variation, however, in the size of the cells.

The effects of nutrition were particularly marked. Production of aerial mycelium was inhibited by a high nitrogen content of the medium. The presence of thiamine favored rapid growth of the cultures and production of sectors.

Various cultures of S. *scabies* isolated from diseased potatoes differed considerably in their pathogenicity. There was no correlation, however, between the pathogenicity and the cultural characteristics of the strain. The variants obtained from a given culture also differed from the mother culture in their pathogenicity to potatoes.

Thomas (421) isolated six physiologic races of S. *scabies* which distinctly differed in pathogenicity on ten different potato varieties or selections. The most favorable sources of carbon for the growth were sucrose, cellulose, inulin, and maltose. Increasing the nitrogen, phosphorus, and potash content of the medium retarded the production of aerial mycelium. Nitrogen and phosphorus were generally favorable for growth; potash tended to retard it. The different races also showed marked variation in their sensitivity to antiseptics and to extracts of the mycelium of certain fungi. Maximum growth and stability were observed on peat soil; mineral soils tended to retard or inhibit growth and increase variability in the races studied. The more pathogenic races were most stable on most media. Some variant types were peculiar to individual races, but certain types were produced frequently by several races, which pointed to a close genetic relationship between those races.

These variations make one wonder, therefore, whether the many species described (298) as causative agents of potato scab represent distinct species or only variants of one type of culture.

Another important economic group of actinomycetes, namely, the organisms producing antibiotics, show marked variation in culture. Several variants were obtained from S. *griseus*. They differed morphologically in formation of aerial mycelium, and physiologically in production of streptomycin, formation of acid, rate of glucose consumption, autolysis, and production of pigment. Intermediary variants were also obtained.

The freshly isolated streptomycin-producing strain of S. *griseus* formed typical aerial mycelium, characteristic of the species. It changed the reaction of a glucose-containing medium to alkaline, produced characteristic types of surface and submerged growth, underwent only limited lysis, and was markedly resistant to the antibiotic action of

streptomycin. On the other hand, the nonsporulating variant produced no aerial mycelium, formed no streptomycin, was sensitive to the antibiotic action of this substance, was characterized by a type of growth that in shaken culture underwent rapid lysis, and produced acid in the glucose-containing medium. Both strains otherwise possessed the various cultural properties which are characteristic of the S. *griseus* species as a whole, such as lack of pigmentation in organic media and proteolytic and diastatic properties. The nonsporulating strain, when isolated as such, however, would hardly be recognizable as typical S. *griseus*. In view of these variations, the question was raised: Is it possible that many of the *Nocardia* species represent degenerate forms of *Streptomyces*?

Another variant of S. *griseus* produced a red-pigmented vegetative growth. This was accompanied by a loss in capacity to produce streptomycin; in its place another antibiotic, pigmented red and active only

Table 8: *Production of streptothricin by two strains of S. lavendulae and their variants* (478):—

Strain or variant	Streptothricin*
Strain No. 8	25
Variant 8a	<2
Variant 8b	50
Strain No. 14	23
Variant 14a	23
Variant 14b	0

* Units of streptothricin produced in shaken cultures, after 4 days incubation.

upon gram-positive bacteria, was formed. This culture if freshly isolated from a natural substrate would definitely not be considered as S. *griseus*.

Another antibiotic-producing organism, S. *lavendulae,* was also found (478) to vary greatly in culture (Table 8). The variants differed in the amount and nature of soluble pigment in peptone-containing media, in the presence and nature of aerial mycelium and in its pigmentation, and in the production of streptothricin. One strain of the organism gave rise to two variants: one producing bluish colored vegetative growth, initially blue diffusible pigment, and a lavender-colored aerial mycelium with a slightly blue tinge; and the other producing cream-colored vegetative growth, a soluble brown pigment in peptone media, and a lavender-colored aerial mycelium. Two variants were also isolated from sectors of colonies of another strain of S. *lavendulae:* one forming a white aerial mycelium, sometimes showing a faint shade of pink; and the other devoid of aerial mycelium, except for a scant growth of sporulating aerial hyphae on some of the old slants. When the ability of these four variants to produce streptothricin in

shaken cultures was compared with that of the original culture, the first variant of the first strain was almost completely inactive, whereas the second variant of that strain was more active than the parent strain, and the strains free of aerial mycelium were completely inactive.

The production of streptothricin by *S. lavendulae* was thus associated with the ability of the culture to form aerial mycelium, similar to one of the variants of *S. griseus*. In both cases, the variants which failed to produce aerial mycelium likewise produced culture filtrates which possessed no antibiotic potency. Aerial mycelium is not, of course, a determinant for the formation of antibiotics, since numerous cultures of both *S. griseus* and *S. lavendulae* which produce abundant aerial mycelium are unable to form the respective antibiotics.

Jones (201) examined 1,298 freshly isolated cultures of *Streptomyces*. About 20 per cent of these showed from the start considerable fluctuation in the production of aerial mycelium, 6 per cent producing only vegetative growth in the first transfer. The question was, therefore, raised: How many representatives of the fluctuating group may be assigned to the genus *Streptomyces* or *Nocardia*?

Culture Constancy:—In the utilization of actinomycetes for the production of antibiotics, it is highly important to be able to depend upon the constant characters of a culture. In view of the fact that a given organism may be subject to a great many variations due to conditions of cultivation and environment, as well as to other conditions, it is essential to be able to come back to the original culture.

With the purpose in view, a given culture is usually inoculated into moist sterile soil. After the culture has made some growth, the soil is allowed to dry out. The culture can thus be kept for a considerable time. When required, the soil is plated out and the culture reisolated (200).

On comparing cultures kept in soil with similar cultures kept in synthetic media, it was found that the latter tended to lose their capacity for producing aerial mycelium and for abundant sporulation (115).

Chapter V

METABOLISM OF ACTINOMYCETES—GROWTH AND NUTRITION; PRODUCTION OF ODORS AND PIGMENTS

Nutrient Requirements:—Actinomycetes vary greatly in their nutrient requirements. Some are able to thrive on very simple compounds, whereas others grow only on highly complex organic materials. Moreover, the same organism may be able to adapt itself to a great variety of nutrients, the amount of cell synthesis depending on the availability of the substrate and on the effect upon the growth of the organism of the secondary changes in the medium resulting from the utilization of the particular substrate.

Beijerinck (28) was the first to point out that certain organisms are capable of deriving their carbon nutrition and energy needs from the simplest compounds found in the atmosphere. Although he designated one particular organism as a bacillus (*B. oligocarbophilus*), he himself noted: "We also found another, rarer species belonging to the genus *Streptothrix* Cohn, with corresponding properties." Lantzsch (251) later demonstrated that even the bacillus of Beijerinck was in reality an actinomyces.

Although Beijerinck doubted the need for S, Mn, and Fe in the medium, he emphasized the importance of N, P, K, and Mg. The medium he used consisted of 0.1 to 1.0 gm KNO_3, 0.2 gm K_2HPO_4, 80 mg $MgSO_4.7H_2O$, 0.5 mg $MnSO_4.4H_2O$, 0.5 mg $FeCl_3.3H_2O$ per liter of distilled water. When such a medium was inoculated with a small quantity of garden soil, and flasks were plugged with cotton and incubated at 23°-25°C., there appeared "a thin, white or feebly rose-colored, very dry film, difficult to moisten." The medium remained clear. The growth of the film continued for months and resulted in the accumulation of considerable amounts of organic carbon. Beijerinck found that either nitrate or ammonium salt could be used as a source of nitrogen. The carbon was not derived from the CO_2, but from the volatile carbon compounds of the atmosphere. Beijerinck ascribed to this organism the property of biologic purification of the air.

Lantzsch (251) differentiated between the nutrition of two variants of the organism: the filamentous or branched form which assimilated CO; the coccus-like or bacillary form, which assimilated aliphatic hydrocarbons.

In contrast to this simple mode of nutrition, the other extreme may be found in a manure pile. When stable manure consisting of animal excreta mixed with bedding is allowed to lie in an open pile, with air freely admitted, rapid decomposition sets in, as can easily be detected by extensive solution of CO_2 and NH_3 and by a rise in temperature. When the temperature reaches 60° to 65°C., numerous white patches can be seen throughout the pile. When slides are buried in such a pile, then removed and stained, actinomyces growth is found in great abundance. Attempts to cultivate these organisms meet with great difficulty, however, largely because the artificial conditions of nutrition do not quite approach the natural nutrients and environment.

The actinomycetes as a group obtain their nutrition between the two extremes illustrated. It is no wonder, then, that a great variety of media have been introduced for the growth of actinomycetes. These media are synthetic and organic in nature. For the purpose of cultivation, especially for determining the morphological and cultural properties of the organisms, synthetic media are commonly used. A number of such media are described in the appendix. For certain purposes, however, organic media are required. This is true particularly in the growth of S. *griseus* for production of streptomycin. In this case, a complex organic substance in the nature of meat extract, yeast extract, corn steep, soybean meal, and others is found necessary for the rapid production of the antibiotic. Although streptomycin and streptothricin can be produced in simple synthetic media, the process is much slower and lower yields are obtained.

Carbon sources.—Under natural conditions, actinomycetes live on a large number of substrates. They are able to utilize a great variety of simple and complex organic compounds as sources of carbon and of energy. These compounds include organic acids, sugars, starches, hemicelluloses and cellulose, proteins, polypeptides and amino acids, nitrogen bases, and many others. Certain actinomycetes can also attack, to a more limited extent, fats, hydrocarbons, benzene ring compounds, and even such resistant substances as lignin, tannin, and rubber. There is considerable selectivity in the utilization of these materials, some substances being consumed far more readily than others. Glucose, maltose, dextrin, starch, glycerol, organic acids, and proteins are the best sources of carbon; these are followed by sucrose and other sugars, by sugar alcohols, and by sugar acids (309). Cellulose is attacked only by certain organisms. Agar also can be used as a source of carbon and energy by some actinomycetes, notably certain strains of S. *coelicolor* (363).

As a rule, actinomycetes prefer proteins to carbohydrates as sources of carbon. This preference is so pronounced that when a protein or a protein derivative, such as peptone, and glucose or another available carbohydrate are present in the same medium, an actinomyces attacks the protein first, not only as a source of nitrogen but also as a source of

carbon, and liberates considerable waste nitrogen in the form of ammonia (Tables 9, 10).

Most media for actinomycetes contain a certain amount of carbohydrate. This is included to enable the organisms to make more extensive growth and to serve as a buffer, since with proteins and protein-derivatives as the only source of carbon, ammonia accumulates so

Table 9: *Decomposition of different amino acids by microorganisms* (472):— 100 ml medium containing 1 per cent of amino acid

Amino acid	Organism	Growth, dry weight	NH_3-N produced
		mg	*mg*
Glycine	*Streptomyces* sp.	59	31
—	*Trichoderma* sp.	50	24
Alanine	*Streptomyces* sp.	126	39
—	*Trichoderma* sp.	80	22
Glutamic acid	*Streptomyces* sp.	169	28
— —	*Ps. fluorescens*	128	29
— —	*Trichoderma* sp.	218	29

rapidly as to make the medium too alkaline for further growth of the organism. That the favorable effect of glucose in increasing the growth of actinomycetes in the presence of peptone or protein is due, partly at least, to the neutralizing effect of the ammonia produced from the peptone by the acid produced from the glucose was known to some of the early investigators of this group of organisms (323). In the case of tyrosin utilization by actinomycetes, glucose had a favorable effect upon

Table 10: *Decomposition of glycine by different microorganisms in presence of glucose* (472):— 100 ml medium containing 1 per cent glycine and 2 per cent glucose

Organism	Glycine-N decomposed	Glucose decomposed	Growth, dry weight	NH_3-N produced
	mg	*mg*	*mg*	*mg*
Ps. fluorescens	26	820	99	3
Streptomyces sp.	36	570	213	19
Trichoderma sp.	49	1,490	804	1

the growth of only those organisms that were able to form, from tyrosin, substances that neutralized the acid produced from the sugar. Certain amino acids, like leucine, were utilized by actinomycetes only in the presence of an available carbohydrate.

Urea can serve only as a source of nitrogen; not of carbon (125, 307). Actually, urea is produced by certain actinomycetes from peptone (160).

Among the organic acids, formic, oxalic, tartaric, and hippuric are unfavorable carbon sources; acetic, citric, and malic are favorable (307). Ethyl alcohol and ethylene glycol (also erythritol and dulcitol) are unfavorable sources; on the other hand, glycerol and mannitol are highly favorable. Starch is an excellent source of carbon for a large number of actinomycetes. Various hemicelluloses, such as mannans, are readily utilized.

The ability of actinomycetes to utilize carbon sources has often been used as an aid in species differentiation, especially among species of *Streptomyces*. Pridham and Gottlieb (348) reported that all species are able to utilize *d*-glucose, *d*-mannose, starch, dextrin and glycerol, but not erythritol, phenol, cresol and the sodium salts of formic, oxalic and tartaric acids. Certain compounds, however, may be utilized by certain species and not by others. This is true of rhamnose, raffinose, xylose, lactose, mannose, dulcitol, inositol and the sodium salts of acetic and succinic acids.

Since the wide interest in the production of antibiotics by actinomycetes has arisen, numerous investigations have been carried out on their ability to utilize carbon sources from the point of view of the production of a particular antibiotic. In the case of streptomycin, for example, pentoses were poor carbon sources; among the hexoses, glucose and mannose were best, the latter being particularly effective when combined with l (−) proline, maltose was the best of the disaccharides; the trisaccharides were inferior; of the polysaccharides, inulin was inferior to starch and dextrin, among the alcohols, mannitol was a promising carbon source; none of the organic acids proved to be suitable (100).

Nitrogen sources.—Actinomycetes are unable to fix atmospheric nitrogen. Proteins, peptones, and amino acids form the best sources of this nutrient for actinomycetes. These are followed by nitrates and ammonium salts. The former is sometimes considered (309) better than the latter, possibly because of the residual effect of the basic ion left from the nitrate as compared with the acid ion left from the ammonium salt, which makes the medium less favorable for the growth of actinomycetes. Ammonium sulfate is utilized better than ammonium chloride. Urea and uric acid are readily utilized and converted into complex organic compounds. Some forms of actinomycetes are able also to use nitrites in low concentrations as sources of nitrogen.

The capacity for decomposing proteins is widely distributed among actinomycetes. This is shown by the fact that the great majority of actinomycetes are able to liquefy gelatin; the only nonliquefying forms so far known are among the nocardias, notably *N. asteroides*. Actinomycetes are able to coagulate milk, and later peptonize it; in fact, peptonization commonly occurs without previous coagulation. Coagulation of the milk is a proteolytic effect, due to formation of specific enzymes, rather than an acid effect. Blood serum is liquefied by many of the species. Complex proteins, such as hoof meal and horn meal, are also

attacked (308). Many forms are able to utilize complex humus compounds of soil, as will be shown later.

Certain organic compounds, such as phospholipids, favor extensive vegetative growth of actinomycetes. When living or heat-killed, washed suspensions of bacteria are used as sources of nitrogen, growth of actinomycetes is not enhanced, even in the case of those forms which have the capacity to bring about lysis of the bacteria. This led Erikson (115) to conclude that only small quantities of nitrogen result from the lysis of bacteria, in fact, little more than those found in autolyzates of suspensions of insusceptible bacteria.

Mineral nutrients.—Among the mineral nutrients, phosphorus, potas-

Table 11: *The utilization of carbon and nitrogen sources by S. coelicolor* (78):—

Carbon source* gm/l	Relative growth†	Relative pigment intensity†	Final *p*H	Nitrogen source‡ 0.106 gm N/l	Relative growth††	Relative pigment intensity	Final *p*H
None	17	0	8.4	None	30	32	6.7
d-Glucose, 10	100	100	7.2	l-Asparagine	100	100	6.7
d-Mannose, 10	202	200	7.0	Glycine	83	36	7.0
d-Galactose, 10	84	97	7.1	*l*-Leucine	76	36	7.0
d-Fructose, 10	79	96	7.0	*l*-Tryptophane	98	82	6.9
d-Xylose, 10	143	121	7.2	Urea	86	51	7.0
l-Sorbose, 10	26	0	8.5	$NaNO_3$	18	0	6.0
l-Arabinose, 10	65	46	5.2	$(NH_4)_2HPO_4$	57	0	5.7
Starch, 10	107	87	7.0	Ammonium acetate	18	0	5.6
Inulin, 10	32	0	8.5	Peptone	146	118	6.8
Trehalose, 10	90	38	8.1	Tryptone	91	106	7.0
Cellobiose, 10	81	95	6.7	Casitone	116	100	7.0
Maltose, 10	62	43	7.3	Pepticase	175	118	6.4
Lactose, 10	105	64	6.8	Cassamino acids	116	129	6.6
Sucrose, 10	34	0	8.4	Sodium casseinate	72	53	6.9
Glycerol, 10	135	170	6.6	Gelatin	106	29	6.4
Mannitol, 10	82	88	7.1	Egg albumin	44	35	6.0
Dulcitol, 10	20	0	8.6				
Sorbitol, 10	27	0	8.2				
Acetic acid, 5	33	33	8.4				
Lactic acid, 5	60	0	8.8				
Fumaric acid, 5	69	0	9.1				
Succinic acid, 5	47	0	8.9				
dl-Malic acid, 10	60	0	8.9				
Tartaric acid, 5	20	0	8.7				
Citric acid, 5	24	0	8.2				
Gluconic acid, 5	82	25	8.2				

* Basal medium (gm/l): asparagine—0.5, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements.

† Dry weight and pigment intensity of glucose control taken as 100.

‡ Basal medium (gm/l): glucose—10.0, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements.

†† Dry weight and pigment intensity of asparagine control taken as 100.

sium, and magnesium are usually required in varying concentrations; the need for sulfur, calcium, and iron is often questioned, although there is no doubt that certain organisms, such as the grisein-producing types, benefit considerably from the presence of iron. Traces of other elements, such as zinc are frequently found to have a marked effect upon the growth of certain organisms.

Although actinomycetes are found only seldom in salt water basins (522), their sensitivity to higher concentrations of different ions may be of interest.

Kober (226) reported that some actinomycetes are able to withstand a high salt concentration, 0.5 to 0.6 molar solutions giving the largest sized colonies, although the smallest number, pointing to the selective action of the salt. He also found, that although $MgSO_4$ is not essential

Table 12: *Metabolic changes and efficiency of carbon utilization of S. lavendulae in aerated cultures* (517):—

	Tryptone medium	Glycine medium
Mycelium, dry weight, *mg*	101	106
Glucose consumed, *mg*	488	782
NH_3-N liberated, *mg*	4	22
Nitrogen compounds deaminated, *mg*	92	162
Lactic acid produced, *mg*	126	58
Volatile acid as acetic, *mg*	4	13
Conversion of glucose to lactic acid, *per cent*	26	8
Conversion of glycine to acetic acid, *per cent*		10
Efficiency of carbon utilization, *per cent*	25	14

for growth, it has a favorable effect in fairly high concentrations. Calcium is not essential for growth, but its lack in the medium has an injurious effect unless magnesium is present. Potassium is injurious in concentrations of 0.5 per cent KCl, which can be neutralized, however, by high concentrations of $MgSO_4$.

Growth and Cell Synthesis of Aerobic Actinomycetes:—In ordinary stationary cultures, actinomycetes produce on the surface of the medium a compact pellicle which may be continuous or which may consist of discontinuous masses of growth or even of individual colonies. Occasionally, growth takes the form of a surface ring along the wall of the vessel. Many species give rise to masses of growth only on the bottom of the vessel or in the form of flakes or colonies throughout the medium and on the bottom. This type of growth is greatly influenced by the nature of the spores, the amount of air admitted, and the agitation of the cultures, which results in breaking up of the mycelium or of the spores, thus initiating fresh growth.

In a submerged state, growth of actinomycetes is usually in the form

of flakes or small colonies. These organisms never produce a diffused type of growth throughout the medium, as do the bacteria. The only reports of causation of turbidity can be traced to some of the so-called bacterial variants. The growth of the actinomycetes can be easily filtered off through paper or with some filter aid, or it can be removed by centrifugation. Those organisms which, like the *Micromonospora*, produce only a limited number of spores, grow much better in a submerged and aerated state than in stationary culture.

The amount of growth produced by actinomycetes depends not only on the nature of the organism but also on the nature of the nutrients,

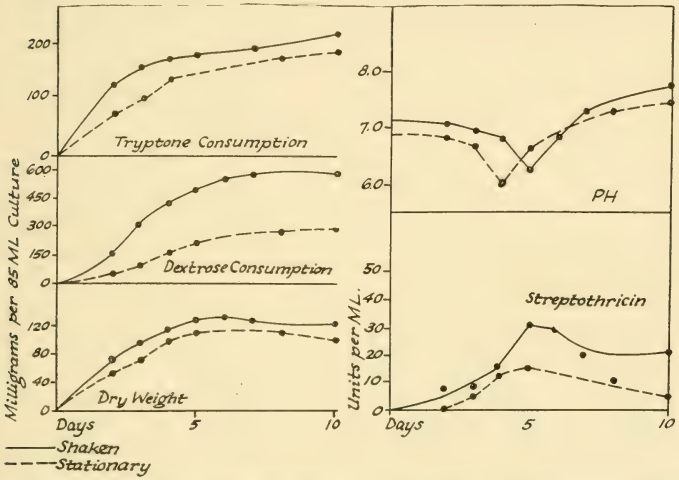

Fig. 18.—Metabolic changes produced by S. *lavendulae* in aerated and stationary cultures (*from* Woodruff and Foster, 517).

their availability, their concentration, and on the environmental factors, such as reaction, buffering of medium, aeration, temperature, stage of growth, and lysis. These factors influence not only the total amount of growth but also the mechanisms of transformation of the constituents of the medium, that is, the physiology of the organisms (Table 11).

In general, there is a definite relation between the concentration and availability of the carbon and nitrogen sources in the medium and the amount of cell material synthesized by actinomycetes. This is brought out in Table 12 and in Fig. 18 (517). The efficiency of carbon utilization is greater in stationary than in submerged cultures. The maximum growth is attained, however, in submerged and aerated cultures. The carbon efficiency of S. *lavendulae* attains 35 per cent in stationary cul-

tures, the corresponding efficiency under submerged conditions being only 21 to 23 per cent (517). As growth progresses, the carbon efficiency drops. After growth of the organism reaches a maximum, less synthesis takes place. If lysis sets in, the mycelium is destroyed and CO_2 and NH_3 are liberated.

The ratio of consumption of carbohydrate to utilization of nitrogen depends upon conditions of growth, nature of organism, and age of culture. With sugar and tryptone in the medium, the ratio increases to about 300 per cent as growth advances, thus pointing to greater oxidation of the carbohydrate in relation to the utilization of tryptone for cell synthesis. This is true especially for submerged cultures, where the abundance of available oxygen leads to greater oxidation of carbohydrate as compared to the tryptone consumed.

Acid production by actinomycetes.—As a result of the growth of actinomycetes in different media, there is always a tendency for the reac-

Table 13: *Acid production by an actinomyces on meat extract-peptone-glucose medium* (340):—

Strain No.	Final *p*H*	Lactic acid
3	8.4	−
4	8.6	−
5	8.5	−
6	4.9	+
7	4.7	++
8	4.8	+
10	5.2	++

* Original *p*H of medium 5.8; 25 days incubation.

tion to become alkaline unless ammonium salts or organic acids are the sole source of nitrogen, with the result that acid ions accumulate in the medium. In the presence of carbohydrates, however, certain organisms are capable of producing certain organic acids, the concentration of the latter depending on the nature of carbohydrate and its concentration. Sooner or later, however, the acid will be decomposed or the organisms will produce neutralizing substances, with the result that the reaction always tends to become alkaline. The tendency is toward the attainment of a maximum alkalinity, which is usually 8.6 to 8.8.

The alkaline reaction thus produced by actinomycetes is largely due to certain secondary reactions in the medium, such as the accumulation of the basic ion (Na, K) when nitrates are used in the medium as sources of nitrogen, or to the formation of ammonium ions from proteins. The fact that certain actinomycetes do not occur in soils having a *p*H lower than 5.2 was at one time considered to substantiate the association of actinomycetes with alkaline reactions. It has now been established, however, that even fairly acid soils contain a considerable num-

ber of actinomycetes (460). Jensen (185) isolated from a forest soil an actinomyces which even had a definite preference for an acid reaction; hence, he named it S. *acidophilus*.

The fact that various actinomycetes are able to produce organic acids form carbohydrates has been long recognized (470). Magnus (281) observed that many of the actinomycetes found in the larynx are able to produce acid of the lactic type (ether-soluble) even in sugar-free media. Plotho (341) confirmed these observations and definitely established the fact that the acid produced by various actinomycetes is of the lactic type, as shown in Table 13.

Woodruff and Foster (517) established that S. *lavendulae* is also capable of producing considerable amounts of lactic acid from carbohydrates. The nature of the nitrogen source is of considerable importance in this connection, as shown in Table 13. In the presence of

Table 14: *Acid formation from glucose in aerated cultures of S. lavendulae* (517):—

Glucose concentration *per cent*	*p*H of medium* after			
	3 days	4 days	5 days	6 days
0	8.2	8.6	8.7	8.8
1	6.8	6.9	7.0	7.4
2	6.5	6.5	6.5	6.5
5	6.2	6.1	5.7	5.7

* Initial *p*H was 7.2.

glycine, for example, much more sugar was consumed but less lactic acid produced than with tryptone as a source of nitrogen. This is particularly true for submerged cultures. In the absence of glucose or with only extremely low concentrations of sugar and in the presence of tryptone, ammonia will accumulate in the medium, gradually making it alkaline. In the presence of 1 per cent glucose, however, the *p*H is lowered appreciably even in buffered media, due to the formation of organic acids. In the presence of 2 per cent glucose, especially in unbuffered media the *p*H levels may go down as low as 3.2 in 2 days. The changes in reaction are thus parallel to the concentrations of sugar (Table 14).

On the basis of the sugar consumed, lactic acid production was found to be equivalent to 25.8 and 7.5 per cent in tryptone and glycine media, respectively. This high conversion took place under conditions of forced aeration. Other actinomycetes, especially S. *griseus*, also produce lactic acid (395). This was found to hold true particularly for the degenerated strains which have lost the capacity to form aerial mycelium.

In addition to lactic acid, S. *lavendulae* produces a certain amount

of a volatile acid, apparently acetic. The volatile acid was believed to be formed by the deamination of the glycine. Large amounts of ammonia also were found to accumulate in the medium as a result of processes of deamination. On the basis of 162 mg of glycine deaminated, the volatile acid, calculated as acetic, amounted to 10.3 per cent of the glycine decomposed.

Oxygen consumption.—S. *lavendulae* oxidizes glucose and glycerol at a very high rate, the oxygen uptake being, respectively, 60 and 45 per cent of the theoretical. The incomplete oxidation is due to assimilation of some of the products for cell synthesis and to the formation of incom-

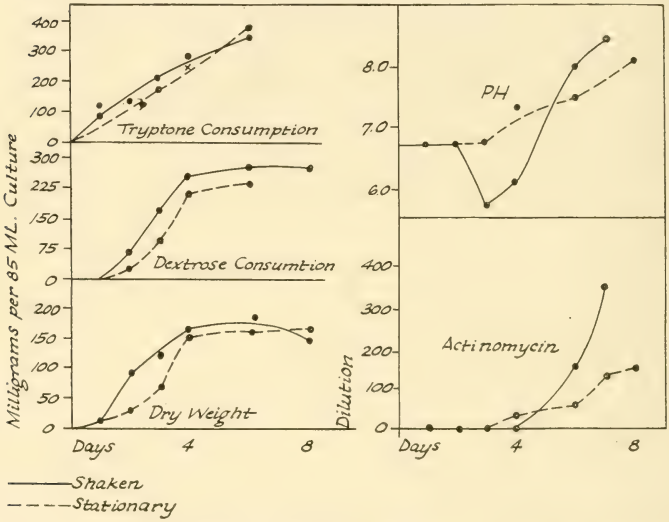

FIG. 19.—Metabolic changes produced by S. *antibioticus* in aerated and stationary cultures (*from* WOODRUFF and FOSTER, 517).

pletely oxidized products, such as lactic acid. When the organism is allowed to starve for 1 to 2 days in a phosphate buffer solution and under aerated conditions, autorespiration will proceed, at a reduced rate, the cells utilizing the reserve cell materials.

Deamination.—Washed cell material of S. *lavendulae*, grown under submerged conditions, was shaken for 18 hours at 30°C. in media containing different amino acids and *M*/30 phosphate buffer at *p*H 6.8, and the relative deamination measured by ammonia formation. The majority of the amino acids were deaminated under these conditions, arginine and histidine being attacked most readily; β-alanine was deam-

inated only about one-third as readily as *d*-alanine. Leucine, isoleucine, and certain other amino acids were not deaminated at all or only in mere traces.

Utilization of complex organic compounds.—It has thus been established that actinomycetes are able to utilize a great variety of organic compounds as sources of both carbon and nitrogen. The nature of the nutrient influences not only growth of the organism, but also its cultural and physiological properties, such as pigmentation, as shown in TABLE 11. Unfortunately, no satisfactory methods have been developed for measuring accurately some of the changes brought about in the decomposition of complex compounds, such as lignins and humus.

Actinomycetes are able to attack numerous other complex organic compounds, such as salicylaldehyde (152), paraffin hydrocarbons (113, 521), rubber (405), and chitinous substances (398).

Reduction of nitrates.—Many actinomycetes possess the capacity of reducing nitrates to nitrites. The importance of this process in the nutrition of the organisms has not been fully established, although a definite parallelism has been observed between growth of the organisms and accumulation of nitrite in the medium. It has also been established that nitrite can be utilized as a source of nitrogen by many actinomycetes, provided its concentration in the medium is not high enough to make it toxic (443, 444).

Nitrate is never reduced to atmospheric nitrogen or to ammonia. Wherever these products have been reported, their formation was due to secondary reactions rather than to direct reduction of the nitrate. Gaseous nitrogen can be formed by interaction of nitrite and amino acids in an acid medium ($2NO_2^- + 4NH_2 \rightarrow 3N_2 + 4H_2O$), a combination quite unlikely in cultures of actinomycetes. Ammonia can be produced in a culture containing nitrate when the synthesized cell material undergoes autolysis.

Influence of environment on growth of actinomycetes.—It is commonly assumed that actinomycetes prefer a neutral or slightly alkaline reaction for their growth, and that they are especially sensitive to a high acidity; many species are not able to grow at *p*H 4.8 (136, 445), as brought out in TABLE 15. The inability of most actinomycetes to grow under acid conditions has been used to advantage in the control of certain plant diseases in the soil, especially potato scab.

The optimum temperature for growth of most of the actinomycetes usually falls between 23° and 37°C. Certain actinomycetes are able to grow at temperatures lower than 20°C. Some organisms prefer temperatures of 20° to 23°C. Still others are thermophilic in nature and are able to grow at 50° to 65°C. The more common forms, however, are readily destroyed at the higher temperatures, the resistance of the spores being only slightly greater than that of the mycelium. When a culture is kept for 10 minutes at 70°C., not only the mycelium but even the spores lose their viability.

TABLE 15: *Influence of reaction on the decomposition of a protein-rich material by actinomycetes* (445):—
100 gm soil + 1 gm dried blood

*p*H OF SOIL	NH_3–N produced in 28 days		
	S. scabies	*S. viridochromogenus*	*S. griseus*
	mg	*mg*	*mg*
3.2	0	0	0
3.6	0	3.1	1.0
4.0	2.2	0.9	1.1
5.0	28.9	9.4	1.3
5.8	68.3	47.5	40.9
6.4	64.3	65.2	60.0
7.2	66.9	63.3	62.0
7.7	62.6	63.6	53.0
8.8	0.4	–	2.8
9.6	0	0	0.4

TABLE 16: *Rate of growth of S. griseus and streptomycin production in shaken cultures* (481):—

Incubation	Growth	*p*H of filtrate	Residual glucose	Streptomycin
days	*gm*		*mg/ml*	*μg/ml*
0		6.8	10.2	0
1	0.048	6.9	9.3	<5
2	0.237	8.5	7.6	5
3	0.394	8.6	5.6	63
5	0.370	8.4	0.5	84
7	0.248	8.7	0.5	62
10	0.140	8.9	0.5	51

TABLE 17: *Metabolic changes produced by S. griseus with different sources of nitrogen* (458):—
Stationary cultures

NITROGEN SOURCE	INCUBATION, DAYS	CELL GROWTH, MG PER 100 ML	SUGAR LEFT, MG PER 100 ML*	AMMONIA NITROGEN, MG PER 100 ML	ACTIVITY μG/ML	*p*H
Sodium nitrate†	5	208	580	–	21	7.6
	11	264	22	–	58	8.3
Ammonium sulfate	5	228	650	55	13	6.2
	11	291	255	53	8	5.6
Peptone	5	212	600	29	38	8.0
	11	365	50	45	80	8.5
Glycine	5	236	550	39	43	8.1
	11	252	45	72	60	8.7

* Control = 980 mg glucose per liter.
† Mineral sources of nitrogen, 3 gm per liter; organic sources, 5 gm per liter.

The optimum temperature for the production of streptothricin by *S. lavendulae* (452) lies between 20° and 28°C.; at 37°C. very little of the antibiotic is produced.

The course of growth of an actinomyces, consumption of energy and metabolic changes, as influenced by different sources of nitrogen are brought out in Tables 16 and 17.

Metabolism of Anaerobic Actinomycetes:—As compared to the aerobic actinomycetes, the anaerobic forms show only limited growth and biochemical activity. According to Erikson, they exert no proteolytic action on egg or serum-containing media; they do not clot or hydrolyze milk and, in fact, rarely grow on it; they seldom grow on gelatin, and when there is a little flaky growth the tubes when cooled are found not to have been liquefied; they have little or no hemolytic action on blood broth or blood agar. Certain strains isolated from human infections have been found to show a slight degree of hemolysis on blood-agar plates at different times, but not consistently. They do not produce soluble pigments on protein media or insoluble pigments in their growth.

Fermentation of sugars by organisms belonging to the genus *Actinomyces* is not accompanied by gas formation. This reaction is fairly constant. Glucose is the most readily fermentable sugar; maltose, lactose, and sucrose come second and are fermented within a comparatively short time by all strains; positive or negative reactions with salicin and mannitol have been found of value in differentiating strains, such as human and bovine (112).

A. bovis was reported by Rosebury (367) to have a limited tolerance for oxygen, which varies, however, among strains. The optimum temperature for this organisim is 37°C., and optimum *p*H is 7.2 to 7.6. Although *A. bovis* grows in the absence of a carbohydrate, it is greatly favored by the presence of glucose. It produces acid from carbohydrates.

A. bovis is killed by heating at 62° to 64°C. for 3 to 10 minutes. Like aerobic actinomycetes, it apparently survives drying for a long time, particularly when kept at low temperatures. Lieske, however, reported (260) that anaerobic forms are very sensitive to drying, being unable to survive even for one day.

Production of Odors:—Most of the aerobic actinomycetes are characterized by the production of a specific odor, which is typical of freshly plowed soil or of composts. This odor is musty, or earthy, and occasionally fruity, in nature. Rullmann (373) believed that the odor is characteristic only of a single species, which he designated as *A. odorifer*. According to Lieske, only those aerobic forms that produce chalky white aerial mycelium with round spores are capable of forming this odor; the nonsporulating forms of the *Nocardia* type and

those that produce cylindrical spores do not give rise to any odor. The presence of carbohydrates in the medium favors odor production. The thermophilic actinomycetes are responsible for the more fruity scents, which arise particularly from young cultures.

Rullmann was the first to make a detailed study of the pungent odor produced by certain species of actinomycetes. The odoriferous substance is soluble in ether (373, 376).

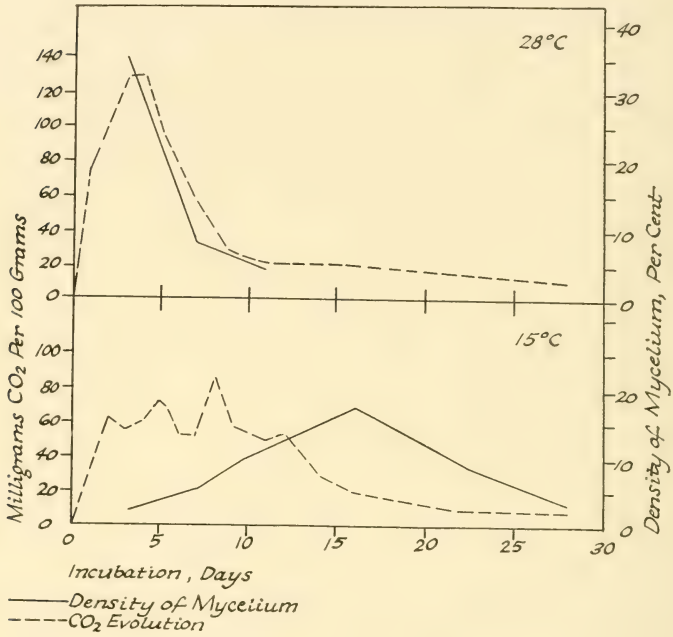

Fig. 20.—Influence of temperature upon growth and carbon dioxide production by actinomycetes (*from* Jensen, 192).

Thaysen (417) found that this substance is partly soluble in ethyl alcohol, and he considered it to be an organic amine. In high concentrations, it had a manurial odor, but in high dilutions, especially in slightly alkaline water, it became markedly "earthy." One strain of an actinomyces was grown in broth, the culture distilled at ordinary pressure and the distillate treated with ether. On removal of ether and dilution of the residual substances 2:10,000,000 in water at *p*H 7.5-8.0, a typical earthy odor was obtained. When the "odor concentrate" was

diluted with water and fish (trout) were placed in it for 1 hour, they absorbed sufficient odor to become markedly tainted and unpalatable (418).

Issatchenko (182) emphasized the importance of the odor imparted to river waters by the actinomycetes in rendering such water unpalatable. In view of the fact that actinomycetes are able to develop at a

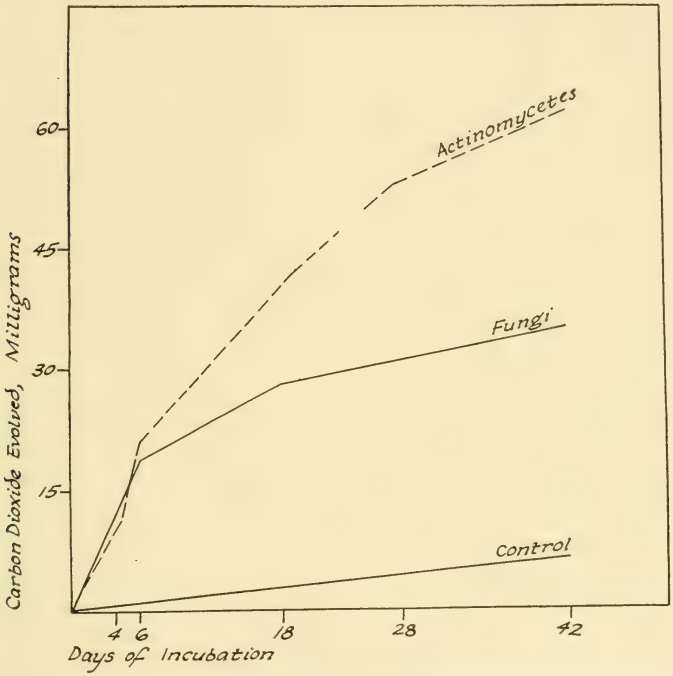

Fig. 21.—Decomposition of hemicelluloses by actinomycetes, as measured by CO_2 evolution (*from* Waksman and Diehm, 462).

much more reduced oxygen pressure than that of the atmosphere (1/25), the large numbers found in the surface layers of the bottom deposits are able to grow rapidly and produce an intense odor. If the bottom is sandy, most of the odor will dissolve in the water; if the bottom is clay, the odoriferous substance will be retained and will accumulate.

Odoriferous substances may also be produced by actinomycetes on cacao beans (55), milk (120), and other foodstuffs, rendering them inferior in quality or totally unsuitable for human consumption.

Production of Pigments:—Actinomycetes are characterized by the production of a variety of pigments both on organic and on synthetic media. Nearly half of all the species isolated and described produce a pigment of one form or another, on one medium or another. These pigments are usually described in terms of various shades of blue, violet, red, rose, yellow, green, brown, black. There are also many gradations of these colors. The nature and the intensity of the pigment are greatly influenced by the composition of the medium and environmental growth factors. The pigment may dissolve into the medium or it may be retained in the mycelium. The pigment is concentrated in the vegetative growth in many cases, and only in the aerial mycelium in others. Certain species produce more than one pigment, as is indicated by such names as *A. violaceus-ruber* and *A. tricolor*. Certain brown shades are often superimposed on the main pigment, especially in organic media.

Some of the pigments are synthetic; others are formed as a result of transformation of certain constituents in the medium. This is true especially of the brown and black pigments produced in protein-containing media, as first shown by Cohn, in 1875, and later studied extensively by Beijerinck and many others.

Production of pigments by actinomycetes has been utilized as an important cultural characteristic in describing the organisms. Nevertheless, the ability to form pigments represents one of the most variable properties among the actinomycetes. This variation depends upon many factors, involving not only the nature of the medium, but also the nature and age of the culture and its previous cultivation. The insoluble types of pigments are more constant than the soluble forms. Acids and alkalies exert a marked effect upon the nature and intensity of the pigment. Some of the pigments are soluble in organic solvents, and others are not.

The production of water-soluble brown to black pigments on organic media is characteristic of certain actinomycetes, mostly members of the genus *Streptomyces*. These organisms have usually been designated as chromogenic forms. The nature and the formation of this pigment were first investigated by Beijerinck (25). The tyrosinase action characteristic of these organisims was believed to explain the mechanism of the production of this pigment. It is insoluble in organic solvents, but soluble in water, in dilute acids, and in alkalies.

According to Afanasiev (6), potato scab organisms failed to produce the melanin pigment in the medium when only tyrosine was present; however, when other nitrogenous compounds were also added, the black pigment was formed abundantly. This was believed to be due to an alkaline reaction that is favorable to the production of the pigment. It was not formed from other amino acids. All plant pathogenic cultures were found to be chromogenic. Although Millard and Burr (298) reported that nonchromogenic actinomycetes may also cause

scab formation, Afanasiev questioned these reports, since the cultures did not cause scab under controlled conditions.

The pigment produced by *S. coelicolor* was first studied by Müller in 1908 (306). This pigment is dark blue and diffuses readily into the medium. If the reaction is acid the pigment becomes red; when the reaction of the medium is alkaline, the pigment is blue. Müller observed that this pigment was produced on synthetic media only with starch as a source of carbon. Waksman, however, demonstrated that this pigment or allied pigments are also produced with sucrose and other carbon sources. Chemically, the blue pigment was at first said (240) to belong to the anthocyanins. This was not confirmed, however, (116). In 1914, Beijerinck (26) described, under the name *A. cyaneus*, a culture which would now be classified with the *Nocardia* group and which produced a pigment similar in its properties to the anthocyanins. This pigment was recently designated as *litmocidin* and was found to possess antibiotic properties (133).

Lieske distinguished two groups of pigments produced by actinomycetes: (*a*) the chromophores or pigment which is not excreted from the mycelium into the medium, and (*b*) the chromopars or pigment which is readily excreted. The first group comprises various pigments produced in the vegetative mycelium grown on synthetic media, namely, yellow, orange, red, blue, violet, brown, black, and green; the aerial mycelium of these cultures may be white, rose, lavender, red, yellow, orange, green, or grey. The soluble pigments are usually yellow, blue, and red; occasionally they are green; and some orange and brown pigments are also produced.

Kriss (240) could not accept Lieske's separation of actinomyces pigments into the above types or the classification of Duché into endopigments and exopigments. Even in the case of the chromophore pigments, part at least of the pigmented material is dissolved in the medium, possibly because of lysis of some of the cells. The solubility of the chromopar pigments in water is due to the greater penetration of the pigment through the cell wall. The chromophore pigments are either insoluble in water and are bound to the proteins or are dissolved in the fats and lipoids of the cell, or they are water-soluble but unable to pass through the living cell plasma; on the death of the cell, the pigment may be able to dissolve into the medium.

Kriss recognized four types of pigments among the actinomycetes:

A. Pigments soluble in water and in 96 per cent alcohol. These pigments are capable of passing through the living cell plasma. They have been subdivided into, (*a*) anthocyanins, soluble only in water, and (*b*) hydroactinochromes, soluble in water and in alcohol.

B. Lipoactinochromes, insoluble in water but soluble in alcohol and in other organic solvents.

C. Pigments insoluble in water and in organic solvents.

D. A combination of water-soluble and water-insoluble pigments.

Only a few of the pigments produced by actinomycetes have been studied from a chemical viewpoint.

Krainsky (230) examined in detail several actinomyces pigments. *S. erythrochromogenus* produced a red pigment soluble in water but not in alcohol, ether, or chloroform. The addition of alcohol to an aqueous solution of the pigment brought about its precipitation. Acids and alkalies had no effect upon it. A yellow pigment was isolated from *S. cellulosae*. It became violet-red in an alkali solution and blue-green in concentrated H_2SO_4. This, as well as the red pigment, was considered to be a carotin. The green pigment of *S. viridochromogenus* was found to change to red on treatment with concentrated H_2SO_4.

Waksman demonstrated that the pigment produced by *S. violaceus-ruber* behaved as an indicator, being red in an acid and blue in an alkali; the change in pigmentation took place at *p*H 6.6 (443). Conn (78) concluded that the two blue pigments produced by two species of *Streptomyces, S. coelicolor* and *S. violaceus-ruber,* are not identical. The pigment produced by the first is similar but not identical to azolitmin. On the basis of this differentiation, Conn believed that the two organisms represent distinct species. This concept could not be accepted by Oxford (330), since the pigment contained too little nitrogen; neither could its phenazine (116) or anthocyanidin nature be accepted.

Lieske studied a carmine-red pigment that became, on boiling in dilute acid, soluble in alcohol and in ether. The brick-red pigment of other strains of actinomycetes becomes soluble only under the action of concentrated HCl; on treatment with H_2SO_4 it is changed to a blue-green pigment. *N. polychromogenes* produces a red pigment, soluble in chloroform, ether, and acid, but not in alcohol, glycerol, water, or dilute alkali; this pigment is also changed to blue-green by H_2SO_4. A light yellow pigment produced by certain actinomyces species was found to be insoluble in organic solvents, but soluble in dilute KOH solution; it changed, on treatment with concentrated H_2SO_4, first to green, then to dark brown. According to Lieske, the green, brown, and violet pigments of the chromophor type are insoluble in common solvents and give a sepia-brown color when treated with concentrated H_2SO_4. The yellow-red pigment of *N. corrallina* was later identified (354) as belonging to the lipochrome group of fat-soluble pigments.

Pigment formation by actinomycetes is influenced by the reaction of the medium, aeration, temperature and by the carbon and nitrogen sources, as shown previously in Table 11. According to Kriss, the composition of the medium has a quantitative rather than a qualitative effect upon pigment production. He measured the adsorption spectrum of the pigment obtained by extraction with ether and alcohol from *S. longisporus ruber*. Although several pigments were thus recognized, they were apparently related. The blue pigments of *S. coeli-*

color could be extracted with cold and hot water as well as with alcohol. This pigment became red when treated with acid, and green when treated with 25 per cent alkali solution. The addition of lead acetate to an aqueous solution of the pigment brought about formation of a violet precipitate. As has been pointed out, this pigment was believed to belong to the anthocyanins, a fact not confirmed (116) by further study.

Krassilnikov (234) confirmed the observations of Kriss, that anthocyanins or allied pigments are characteristic of several actinomycetes. The pigment of *N. cyanea* is soluble in water and in aqueous solutions of alcohol, but not in pure alcohol, acetone, ether, or chloroform. It does not change in color in an acid medium, although in dilute acids the pigment assumes a rose-violet shade; strong acids decolorize the pigment. It is produced only on synthetic media with sucrose and glucose as carbon sources.

Green actinomycetes also produce a water-soluble green pigment, which is the reason for such species names as *A. viridis, A. viridochromogenus,* and *A. verne.* The pigment is also soluble in glycerol and in alkali solutions, but not in organic solvents.

The water-insoluble pigments have been studied only to a limited extent. Among these, the carotenoids produced by the red, orange, and yellow species are of particular interest (234). Reader (354) demonstrated two such pigments among actinomycetes; one of these pigments was designated as corallin, an ether solution of which gives two bands of absorption in the spectrum.

The significance of the various pigments, especially the brown and black types, in the nutrition of actinomycetes is still a matter of speculation. Schibata (396) suggested that they play a role in the oxygen exchange between the atmosphere and the cells in a manner similar to the role of hemoglobin in animals. Protective mechanisms have been postulated for some of the pigments (234).

Thermophilic Actinomycetes:—Among the thermophilic microorganisms, or those capable of growing at higher temperatures, such as 50° to 65°C, the actinomycetes occupy a prominent place. In view of the fact that these organisms occur so abundantly in organic matter-rich materials, one would naturally expect that they should be abundant in high temperature in heaps of hay, composts, and in soils, as will be shown later (p. 144). They are also found in a number of other substrates, such as pasteurized cheese (36).

The abundance of thermophilic actinomycetes in nature has been known since the work of Globig (138), in 1888. Tsiklinsky (429) was the first to establish, in 1899, that composts contain an abundance of actinomycetes. The normal temperature for their growth ranges from 50° to 70°C.

Thermophilic actinomycetes in culture can be isolated by one of several simple procedures. Tsiklinsky inoculated sterile potatoes with compost material and incubated them at 53-55°C. After 16 hours' incubation, plates were prepared and incubated at the same temperature. Two cultures of actinomycetes were thus obtained, one of which produced chains of spores and may, therefore, be considered as a species of *Streptomyces*, and the other produced round or ovoid spores at the end of side branches, caused by the swelling of the tips, thus representing a true *Micromonospora*. The second organism was believed to be widely distributed in nature and was designated as *Thermoactinomyces vulgaris*. It grew at 48-68°C., with an optimum at 57°C. At 37°C. or at lower temperatures, it remained inert, but became active within 24 hours when incubated at 56-57°C. The spores of this organism were not destroyed at 100°C. even after 20 minutes. The organism also resisted the action of disinfectants and grew readily on most of the ordinary media. It was strongly proteolytic but not amylolytic. The Streptomyces form, designated *Thermoactinomyces* II, was less proteolytic, and the spores were less resistant to heat.

Gilbert (135) isolated several thermophilic actinomycetes from various soils. He included them under one species as *A. thermophilus*. The organisms produced a lichnoid growth, with white aerial mycelium which later became gray. The optimum temperature for growth was 55°, with a maximum at 60°C. Most strains ceased to grow even at 45°, although some could be adapted to grow on agar media at 37° and even lower temperatures. Gelatin was slowly liquefied.

Miehe (295) looked upon the thermophilic actinomycetes as the characteristic organisms inhabiting the decomposing masses of plant material under high-temperature conditions. These hot composts, rather than the soil, were believed to be the natural substrates of the thermophilic organisms. The spores lost their vitality rapidly, especially on agar media, but survived on hay particles. One organism, designated as *A. thermophilus* Berestneff, grew well at 40°—50° C., more slowly at 30°, and not at all at 25° and 60°C. The manner of spore formation of this organism suggests that it was also a member of the *Micromonospora* group. Miehe reported, however, that some of the thermophilic actinomycetes produced spores in a manner similar to that described by Gilbert. Schütze (399) reported the presence in decomposing clover hay of representatives of two types of thermophilic actinomycetes, one of which was designated as *A. thermophilus* Berestneff and the other as *A. monosporus* Lehmann and Schütze. The latter may be definitely considered a member of the *Micromonospora* group.

In a more recent review of the literature (36) on the occurrence of thermophilic actinomycetes, some 20 species were listed, namely:

Organism:	Author:	Organism:	Author:
Thermophilic forms	Globig	*Thermomyces lanuginosus*	Miehe
Cladothrix thermophile	Kedzior	*Actinomyces monosporus*	Schütze
Thermomyces lanuginosus	Tsiklinsky	*Streptothrix* No. 8	Bruini
Thermoactinomyces vulgaris	Tsiklinsky	*Streptothrix* No. 9	Bruini
		Streptothrix No. 12	Bruini
Streptomyces sp.	Tsiklinsky	*Actinomyces spinosporus*	Velich
Streptothrix thermophile, No. 12	Tsiklinsky	*Actinomyces thermodiastaticus*	Bergey
Streptothrix thermophile, No. 20	Tsiklinsky	*Actinomyces nondiastaticus*	Bergey
Actinomyces sp.	Sames	*Streptothrix thermophilus*	Eckford
Actinomyces thermophilus	Gilbert	*Actinomyces thermophilus*	Krohn
Actinomyces thermophilus	Miehe	*Actinomyces casei*	Bernstein and Morton

Chapter VI

PRODUCTION OF ENZYMES AND OF GROWTH-PROMOTING SUBSTANCES

Actinomycetes are able to produce a variety of agents which are essential to their own growth or to that of other organisms living in association with them. Some of these substances are of the nature of enzymes, others are vitamin-like substances, and still others are lytic agents. Actinomycetes also produce a variety of bacteriostatic and bactericidal substances, or antibiotics, which are discussed in Chapter VII.

Production of Enzymes:—Actinomycetes produce both extracellular and endocellular enzymes. Only very few of these enzymes have been concentrated and studied in detail. The presence of others has only been demonstrated in the culture medium or in the mycelium of the organism.

Proteases.—The wide distribution of proteolytic enzymes among actinomycetes is indicated by the ability of the organisms to liquefy gelatin with different degrees of rapidity and to attack serum protein, coagulated egg-albumin, casein, and vegetable proteins (442). This property has been utilized for species characterization. In nearly half of the species, especially those belonging to the genus *Streptomyces*, gelatin liquefaction is accompanied by production of a brown pigment. Optimum gelatin liquefaction occurs at a *p*H of 6.5 to 8.5. Greater acidity is more injurious to the proteolytic process than is a more alkaline reaction.

The proteolytic action of actinomycetes, in contrast with that of fungi and bacteria, is not influenced to any great degree by the presence of glucose or other available carbohydrates. The breakdown of the protein proceeds, through the amino acid stage, to ammonia. This is brought out in Table 18. In some cases it is easy to establish the intermediary formation of peptides and amino acids; in other cases, it is more difficult.

The proteolytic enzymes are fairly resistant to the effect of temperature, since they are able to withstand heating at 70°C. for 30 minutes. According to Lieske, the resistance of the enzymes to the effect of higher temperature is greater than that of the living cells of the organisms, the latter being killed at 62°-65°C. When the enzymes are

heated to 80°C., their activity is destroyed. When some of the cultures are kept for a long time at 40°-45°C., the reproductive capacity of the cells is destroyed, but their proteolytic functions are not injured (234). The ability to cause proteolysis is more marked for the non-pigmented forms than for the pigmented types. Beijerinck postulated that the pigmented forms are responsible for a solidifying effect produced by the quinone upon the liquid gelatin. To what extent this is responsible for the apparently lower proteolytic action of pigmented cultures still remains to be determined.

Table 18: *Proteolytic activity of actinomycetes in* 2 *per cent gelatin solution* (187):— Results in milligrams of nitrogen per 25 ml of medium

Species	10 days		30 days		Liquefaction of gelatin
	NH_4-N	Formol titration	NH_4-N	Formol titration	
S. griseus	6.4	16.5	16.2	30.7	Very rapid
S. griseoflavus	7.1	17.1	22.1	36.6	Very rapid
S. cellulosae	5.0	16.0	14.5	27.4	Rapid
S. olivaceus	2.3	13.2	9.2	24.0	Rapid
S. fulvissimus	2.5	11.8	7.0	29.5	Fairly rapid
S. violaceus-ruber	2.6	14.5	9.2	39.2	Rapid
S. roseus	2.2	11.4	10.2	20.2	Slow
S. bobiliae	2.3	11.2	6.2	26.9	Slow
S. viridochromogenes	2.2	11.1	10.6	19.8	Slow
S. erythrochromogenes	3.5	13.0	7.7	20.5	Slow
S. phaeochromogenus	2.0	9.3	4.4	22.3	Slow
S. diastatochromogenes	3.1	11.2	11.1	21.5	Slow
S. aureus	2.1	8.8	5.8	22.6	Slow
Sterile solution	–	–	0.0	6.8	–

In some species, proteolysis occurs only at a late stage in the development of the organism. This may be due to the formation of endoenzymes, which are liberated on the death of the cells, as contrasted with the exoenzyme produced at an early stage of the development of the mycelium. This explanation has not been universally accepted, however (234). Bacteriolysis may often parallel growth inhibition (Table 19).

The ability of certain actinomycetes to cause the lysis of various bacteria is characteristic of certain species of *Streptomyces*. This effect directed upon plant pathogenic bacteria may be of considerable economic importance. A culture designated as *Streptomyces* 105 which produced a wine-colored soluble pigment and a white to gray aerial mycelium was found (83) to exert a lysogenic effect upon various species of *Phytomonas, Erwinia,* and other gram-positive and gram-negative bacteria. Potato extract-glucose agar media were particularly favorable to the production of the lytic agent. Cultures of *Streptomyces* 105

added to soil infected *Phytomonas tabaci* protected the plants against infection. The importance of such lytic agents in soil processes and their relation to true antibiotics are matters for further investigation.

The ability to coagulate milk and to dissolve the coagulum is also a common property of actinomycetes. Whether this is due to the formation of a special enzyme of the nature of rennet or lab or whether it is a property of the proteolytic mechanism of the organisms remains to be determined.

Many strains of actinomycetes are able to hemolyze blood cells, as a result of production of hemolysins. These enzyme systems are distinct from the true proteolytic enzymes, and are also fairly resistant to heat. They apparently have no relation to the pathogenic properties of the organisms producing them.

Table 19: *Distribution of bacteriolytic properties among actinomycetes* (503):—

Preparation	Total strains	Activity against *S. aureus*			Growth-inhibition of *B. subtilis*		
		++	+	0	++	+	0
Number of strains	164	24	54	86	22	54	88
Per cent of strains	100	14.6	32.9	52.4	13.42	32.9	53.7
Number of filtrates	67	9	11	47	5	4	58
		Activity on heat-killed *E. coli* and *S. aureus*					
Number of filtrates	67	23	25	19	–	–	–

The species of *Nocardia* are usually much weaker proteolytic forms than the *Streptomyces* species. Some of the nocardias, notably the red and green types, do not liquefy gelatin at all. This is true of *N. asteroides,* an important pathogen, and of the saprophytic *N. ruber, N. viridis,* and others. Some of the yellow species (*N. flava*) are weak liquefiers. The lemon-yellow forms (*N. citrea*) and the white types (*N. alba*), however, liquefy gelatin energetically.

While the proteolytic property of actinomycetes is a constant characteristic, it is essential to remember that the occurrence and the rate of proteolysis, as measured by the extent and rapidity of the reaction, are influenced by environment and are variable properties.

Although no enzyme preparations comparable to those of certain fungi and bacteria are obtained on a large scale from actinomycetes, there is no doubt that such preparations could easily be obtained. Whether they would have any distinct advantages is hard to tell. Possibly the thermophilic capacity of some of the organisms might yield enzymes which would be more heat-tolerant than those produced by fungi and bacteria.

Certain actinomycetes exhibit, for example, marked proteolytic activity upon wool fabrics. The sterile culture filtrate of one organism was found (141) to exert a marked effect not only upon protein derived from soybeans, casein, peanut and corn, but also upon wool fiber, to the extent of 5 to 85 per cent.

Amylases.—A large number of actinomycetes are capable of bringing about rapid hydrolysis of starch, either to the dextrin stage or directly to maltose and glucose. These reactions are carried out by means of active amylolytic enzymes. This phenomenon was first observed by Beijerinck and Sames (377), and later confirmed and extended by Krainsky, Waksman, Lieske, and others.

The method of screening a large number of cultures consists in streaking agar media containing starch as the source of carbon, and allowing it to incubate. After 5, 10, 15 and 20 days, the surface of the agar is covered with a solution of I-KI, and the degree of starch hydrolysis measured by the width of the clear zone. It is thus possible not only to establish that many species are capable of producing amylolytic enzymes, but the active forms can be selected for further study. Formation of a zone of 1.0 to 1.5 cm. in 10 days is an index of excellent amylase production. Inorganic sources of nitrogen, especially nitrates, appear to be preferable to organic forms for the production of amylolytic enzymes.

Just as in the case of the proteolytic enzymes, the amylases of actinomycetes are able to withstand the effect of higher temperatures better than are the cells of the organism producing these enzymes. Surovaya (410) obtained a potent diastatic enzyme preparation from a culture of an organism described as *S. diastaticus*. The culture was grown on a potato medium for the production of the enzyme. The preparation was designated as "superbiolase." It was active at 70° to 100°C. and had an optimum *p*H at 6.6 to 6.7. The starch was converted first from the insoluble into the soluble form and then to dextrin. Saccharification of the dextrin proceeded much more slowly than starch liquefaction. This points to the possible application of such enzyme preparations to industrial processes where the sugar produced is not essential.

Many species of actinomycetes are also able to attack dextrins, glycogen, and inulin and to produce the corresponding enzymes. So far, however, no attempt has been made to study these enzyme systems in detail or to utilize them for any practical purposes.

Invertase.—The wide distribution of invertase among the actinomycetes has been pointed out by Krainsky, Caminiti (61), and Waksman. The ability of some species to utilize sucrose as a source of carbon is dependent upon the property of the organisms to produce this enzyme.

Although many species of *Streptomyces* and *Nocardia* are able to utilize sucrose, the production of invertase has not been established for all forms. It has even been suggested that this property be utilized for

differentiating species; at best, however, this can be only a secondary characteristic.

Cellulolytic enzymes.—Although many actinomycetes are able to grow on cellulose as the only source of carbon (231), the production of corresponding enzymes has so far not been demonstrated.

Lipase.—The ability of various actinomycetes to produce lipolytic enzymes has been established (260). The activity of these enzymes upon natural products is often accompanied by the formation of odoriferous substances, discussed in Chapter V. The spoilage of cacao by certain species of *Streptomyces* (55) may possibly be due to the lipolytic effect combined with odor production.

Bacteriolytic and autolytic enzymes.—The ability of certain actinomycetes to dissolve the dead and in many cases also the living cells of many bacteria has been ascribed to the action of specific lytic enzymes or bacteriolysins. This phenomenon was first observed by Lieske, and later studied extensively by Gratia (153, 155), who utilized this process for the preparation of certain bacterial vaccines, such as typhoid vaccine. The bacteriolytic substance produced by S. *albus* was designated by Welsch (505) as "actinomycetin." This property is widely distributed among the actinomycetes, as shown in Table 19; as many as 50 per cent of all cultures have been found active against heat-killed cells of *E. coli* and against living S. *aureus.*

Borodulina (43) and Nakhimovskaia (316) found that among actinomycetes the lytic principle is excreted by the cells into the medium, thereby inhibiting growth of bacteria found in proximity to the lytic principle and, later, dissolving these bacteria. Though resistant to heat, this substance was still considered as an enzyme. Krassilnikov believed that this bacteriolytic enzyme is similar to lysozyme of animal origin, although marked differences have been established between the action of this agent and that of the lytic principle of actinomycetes. Among these antibiotic preparations obtained from this group of organisms, two appear to have properties which would place them either with enzyme systems or with true antibiotics. These are actinomyces lysozyme and actinomycetin. Some of these lytic systems consist of a lipoidal bactericidal substance, a ribonucleinase, and proteolytic enzymes (407). The ability of certain specific phages to attack actinomycetes has been discussed previously (p. 62).

Production of Vitamins:—The favorable effect exerted by certain actinomycetes upon the growth of various fungi was believed (171, 280) to be due to their ability to synthesize thiamin, which is produced on simple synthetic media. A study has been made of 22 cultures of actinomycetes grown in thiamin-free media; this was followed by the inoculation of the same cultures with *Phycomyces blakesleeanus.* The fact was established that all the cultures produced thiamine or its intermediate or its precursor. The production of carotinoids by certain ac-

tinomycetes has been demonstrated, as mentioned previously (p. 97). The ability of certain strains of S. *griseus* to produce vitamin B_{12} has also been established (p. 191).

Oxidative Mechanisms:—Actinomycetes possess a number of oxidative mechanisms, only few of which are recognized at the present time. Attention may be called, for example, to the ability of "resting cells" of certain species of *Streptomyces* to transform aestradiol to oestrone (507). According to Turfitt (430, 431), various species of *Nocardia* are capable of attacking various steroids, with the possible exception of halogen-substituted derivatives. The oxidation of cholesterol results in the formation of a cholesterone, followed later by molecular fission, the products of which may be utilized by the organisms for their further growth. Various actinomycetes are also capable of producing penicillinase (506).

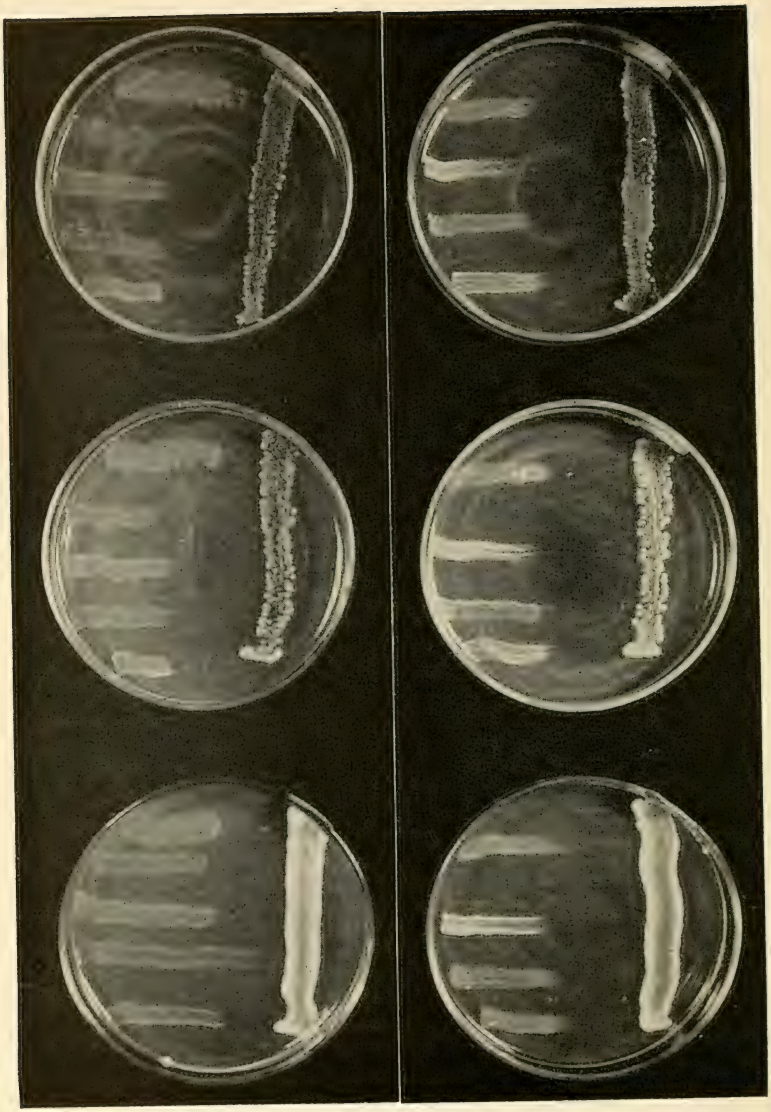

FIG. 22.—The use of the agar cross streak method for testing the ability of actinomycetes to produce antibiotic substances.—Upper two plates, *S. lavendulae* 3516; middle two plates, *S. lavendulae* 3440; lower two plates, *S. griseus* 3496.—Test bacteria, from top to bottom: (a) right column, *M. ranae*, *M. avium*, *M. tuberculosis* 607, *M. tuberculosis* 607R; (b) left column, *B. mycoides*, *B. subtilis*, *E. coli* W, *E. coli* R, *S. aureus*. (R = forms resistant to streptomycin.) (*Original.*)

Chapter VII

ANTAGONISTIC PROPERTIES OF ACTINOMYCETES AND PRODUCTION OF ANTIBIOTICS

Antagonistic Effects of Actinomycetes:—Actinomycetes comprise a large number of organisms which have the capacity of inhibiting the growth of and even destroying other microorganisms, namely, bacteria, fungi, and other actinomycetes. Several detailed reviews of this phenomenon have been published during the last decade (448, 449, 451, 453).

Any student of soil microorganisms who uses the plate method for counting purposes has observed that some of the colonies of actinomycetes on the plate are surrounded by clear zones free from the growth of bacteria and fungi (483). In 1917, Greig-Smith (157) observed, for example, that when a soil is plated out on a nutritive agar the growth of certain spreading colonies of *B. mycoides* and *B. vulgatus* may be inhibited by other colonies on the plate. These will be surrounded by a clear zone 2 to 10 mm. wide where the spreader does not penetrate. Examination of the colonies that produce this toxic effect showed them to consist of actinomycetes. Further study of the various types of colonies brought out the fact that the nonchromogenic strains produced the most toxic effect. He postulated, therefore, that the ability of actinomycetes to antagonize bacteria and fungi may suggest their possible importance in the soil as a factor which limits microbial development and thus affects soil fertility processes.

Although the soil may thus be considered to be a source of antagonistic actinomycetes (488), the enrichment of soil with specific pathogenic bacteria, such as *M. tuberculosis* does not necessarily lead to the development of specific actinomycetes active upon such bacteria (480, 489). The reason for this is that the growth-inhibiting effect of actinomycetes upon bacteria and fungi is brought about largely through the production of toxic agents, which are now known as "antibiotics." The production of such substances can easily be demonstrated for some organisms by the agar-cross-streak method. In many cases, however, organisms that show inhibition of bacteria on the plate do not produce any antibiotic substance when grown in liquid media.

Gasperini (130) was the first to demonstrate the antagonistic action of actinomycetes. He observed that these organisms develop on fungus mycelium, upon which they live to a limited extent in the form of a

parasite, as a result of the faculty that the actinomycetes possess of digesting the membrane of these lower fungi. Greig-Smith first demonstrated the ability of actinomycetes to produce antibiotic agents.

Lieske, who tested a large number of actinomycetes for their antibacterial action, established that this process is selective in nature, affecting only certain bacteria, such as *S. aureus* and that different actinomycetes vary greatly in this respect. Lieske believed, however, that the antagonistic effect of actinomycetes may be due to a specific bacteriolytic enzyme, namely: "Ein bestimmtes bakterienlösendes Enzym könnte aus den Kulturen nicht isoliert werden; dass ein solches in Frage kommt, ist aber bei der grossen biologischen Bedeutung, welche die Vernichtung von fremden Mikroorganismen in der Natur für die Strahlenpilze besitzt, nicht ausgeschlossen."

Rosenthal (369) introduced, in 1925, suitable methods for measuring bacteriostatic and bacteriolytic activities of actinomycetes. He isolated from the dust an actinomyces culture which he designated the true biological antagonist of the diphtheria organism. The surface of an agar plate was covered with an emulsion of the test bacteria, and the actinomyces culture was inoculated into several spots on the plate. After 2 days the actinomyces colonies were surrounded by large transparent zones, whereas the rest of the plate was covered with the growth of the diphtheria organism. In another experiment, the agar was mixed with a heavy emulsion of the diphtheria organism, which had previously been killed by heat, and the mixture poured into the plates. After solidification of the agar, the actinomyces culture was inoculated into several spots on the plates. The actinomyces colonies gradually became surrounded by clear zones, thus establishing the fact that the organism produced a lytic substance which diffused through the agar and dissolved the dead diphtheria cells.

Gratia (155) made a careful study of actinomycetes as agents producing materials (mycophages) that are capable of bringing about the lysis of bacterial cells. These effects were largely exerted upon dead bacteria, although living cells were later found to be affected also (154). The antibiotic substance produced by one of the organisms (*A. albus*) at first considered to be of the nature of an endo- and exo-bacteriolysin (499). It was later designated by Welsch as actinomycetin, as pointed out previously. The lysis of living bacteria was considered to occur in two stages: first, bactericidal effect of the substance upon the living bacteria; second, bacteriolytic action upon the dead bacteria, this process being helped by cell autolysis (502, 503).

The first detailed survey of the distribution of antagonistic actinomycetes in nature was made by Nakhimovskaia (316). Of 80 cultures isolated from a variety of soils, 47 possessed antagonistic properties; however, only 27 of these were found capable of liberating antibiotic substances into the medium (Table 20). These actinomycetes possessed the property of inhibiting the growth of gram-positive bacteria

but not of gram-negative bacteria or of fungi. These antibacterial properties were manifested, not only in artificial culture media, but also in the soil. Some of the cultures that were antagonistic to bacteria in nutrient media were ineffective, however, in the soil. The effects were more intense in light, or podzol, soils and much weaker in heavy, or chernozem, soils. The high content of organic matter in the latter types of soil was believed to be one of the factors that resulted in a decrease in the antagonistic activities of these organisms. When the actinomycetes were allowed to multiply in the soil before inoculation with bacteria, the antagonistic effect was very pronounced even in the presence of a high concentration of organic materials.

According to Borodulina (43), actinomycetes are able to antagonize various spore-forming bacteria and bring about the lysis of the living cells. He found that a thermostable substance was produced on

Table 20: *Occurrence of antagonistic actinomycetes in different soils* (316):—

Nature of soil	Total number of strains tested	Number of antagonistic strains	Strains producing antibiotics
Chernozem	24	10	9
Podzol	11	7	3
Solonets	4	4	4
High altitude soil	9	6	5
Sandy soil	6	5	1
Dry desert soil	5	4	1
River bank meadow	14	7	2
Cultivated soil	7	4	2
Total	80	47	27

agar media. The activity of this substance was greatly reduced at an alkaline reaction but was favored by an acid reaction. When *B. mycoides* and an antagonist were inoculated simultaneously into peptone media, no inhibitive effect was produced because the bacterium changed the reaction of the medium to alkaline, thereby making conditions unfavorable for production of the antibiotic substance by the antagonist. When the antagonist was allowed to develop in the medium before the bacterium was inoculated, a strong antibiotic effect became evident in elongation of the vegetative cells of *B. mycoides*. This was due to a delay in fission and was accompanied by the suppression of spore formation.

Krassilnikov and Koreniako (237) also reported that many species of actinomycetes, notably members of the genus *Streptomyces*, but not of *Nocardia*, produce a substance that is strongly bactericidal to a variety of organisms. This substance was said to be particularly active against nocardias, mycobacteria, and micrococci. It was less active upon spore-forming bacteria and had no action at all on non-spore-forming bac-

teria. Under the influence of this bactericidal factor, the microbial cells were either entirely lysed or were killed without subsequent lysis. The action upon spore-forming bacteria was bacteriostatic rather than bactericidal (238). The antibiotic substance studied by these and other Russian workers was believed to be similar to lysozyme.

An attempt to isolate an antibiotic substance from some of the soil actinomycetes was made by Kriss (243). This substance was insoluble in ether, petroleum ether, benzol, and chloroform, and was re-

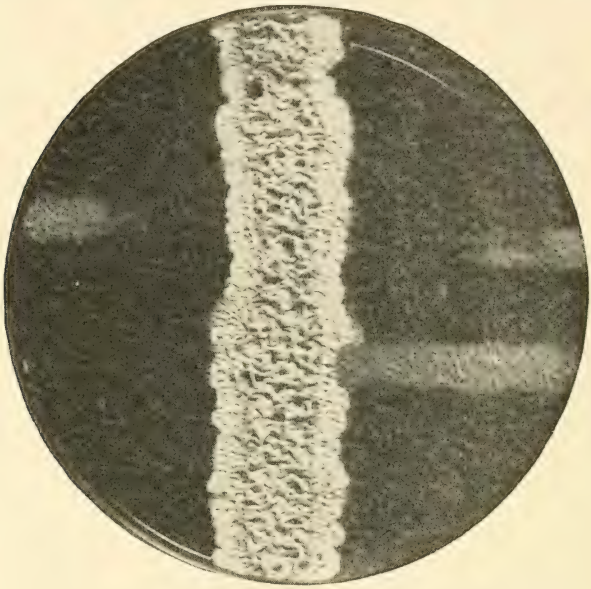

Fig. 23 a.—The use of *M. tuberculosis* for testing production of antibiotic substances by actinomycetes. Upper horizontal streak H37Rv strain; lower streak H37RvR (resistant to > 1000 μg/ml streptomycin): inhibition of streptomycin-sensitive but not of streptomycin-resistant strain (*from* Williston *et al.*, 510).

sistant to the effects of light, air, and high temperatures. The optimum reaction for its production by *Streptomyces violaceus* was found to be *p*H 7.1 to 7.8, the activity not being increased by selective cultivation. Although it was believed that the substance is similar to egg-white lysozyme, the above properties hardly justify this conclusion. The differences in the antibiotic properties of the various antagonistic actinomycetes isolated by the Russian investigators definitely point to the fact that more than one antibiotic substance was involved.

Waksman *et al.* (468) came to the conclusion that actinomycetes possessing antagonistic properties against bacteria and fungi are widely distributed in nature, especially in soils and in composts. Two hundred and forty-four cultures were isolated at random from different soils. Of these, 106 cultures or 43.4 per cent possessed some antagonistic properties, and 49 cultures or 20 per cent were highly antagonistic. Similar relations were observed in examining a large series of well-identified organisms kept for a number of years in a type culture col-

Fig. 23 b.—No inhibition of streptomycin-sensitive or of streptomycin-resistant strains (*from* Williston *et al.*, 510).

lection (503). The antagonistic forms were most abundantly represented by the genus *Streptomyces* (Table 21).

Burkholder (56) examined the antagonistic properties of 7,369 cultures of actinomycetes isolated from soil, using various test organisms, namely gram-positive and gram-negative bacteria, acid-fast bacteria, fungi including yeasts and green algae. Of these cultures, 1,869 inhibited *S. aureus* in agar streak plate tests, 261 inhibited *E. coli*, and 514 showed an antagonistic effect against *Candida albicans*.

Various other surveys have been conducted dealing with the capacity of large numbers of actinomycetes to inhibit the growth of bacteria as

a whole, of certain groups of bacteria (249), of fungi pathogenic to man (391), of viruses (196), and of phages (392, 393).

The antagonistic properties of actinomycetes are not limited to members of the genus *Streptomyces*. A culture of *Nocardia*, isolated by Gardner (129) as an air contaminant, was found to produce antagonistic effects against a variety of gram-positive bacteria. The active substance produced by this organism was designated as *proactinomycin*. A representative of the genus *Micromonospora* was also reported (503) to be capable of exerting antagonistic effects against certain bacteria.

Actinomycetes also reveal antagonistic activities against fungi (424).

Table 21: *Distribution of antagonistic actinomycetes in nature* (468):—

Source of organisms	Number of cultures isolated	Group I*		Group II		Group III		Group IV	
		No. of cultures	Per cent of total	No. of cultures	Per cent of total	No. of cultures	Per cent of total	No. of cultures	Per cent of total
Fertile, manured, and limed soil	74	20	27.0	5	6.8	1	1.3	48	64.9
Infertile, unmanured, limed soil	75	11	14.7	8	10.7	4	5.2	52	69.3
Potted soil	13	1	7.7	1	7.7	0	0	11	84.6
Potted soil, enriched with *E. coli*	21	1	4.8	4	19.0	4	19.0	12	57.2
Potted soil, enriched with mixtures of bacteria	15	12	80.0	2	13.3	0	0	1	6.7
Lake mud	9	3	33.3	4	44.4	0	0	2	22.2
Stable-manure compost	37	1	2.7	20	54.0	4	10.8	12	32.4
Total	244	49	20.1	44	18.0	13	5.3	138	56.6

* I—Most active antagonists; II and III—more limited antagonistic properties; IV—no antibacterial effects with methods used.

Alexopoulos (8, 9) made a survey of the antagonistic properties of 80 cultures of actinomycetes, using *Colletotrichum gloeosporioides* as the test organism. The following results were obtained: 17.5 per cent of the cultures were strong inhibitors, 38.8 per cent were weak inhibitors, and 43.7 per cent had no inhibiting effect upon the fungus.

Meredith (291) surveyed the distribution of organisms antagonistic to *Fusarium oxysporum cubense* in Jamaica soils. Most of these antagonists belonged to the actinomycetes. The antagonists were not evenly distributed in the various soil samples, 10 of the 66 samples yielding 44 per cent of the antagonistic organisms. Those actinomycetes

that were antagonistic to the *Fusarium* when grown in their own soil-infusion agar were not always antagonistic when tested in soil-infusion agar prepared from other soils. A culture of an actinomyces isolated from a compost produces lysis of *Fusarium*. When spores of both organisms were mixed in an agar medium, the fungus at first developed

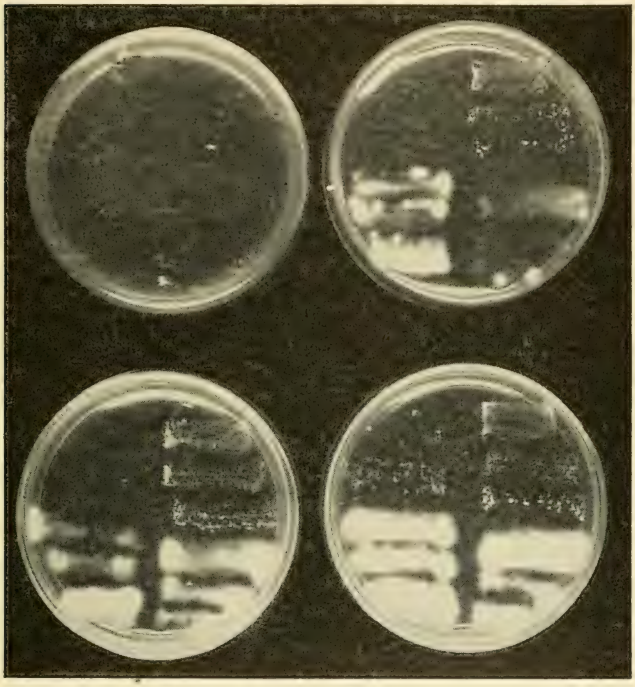

Fig. 24.—Method of measuring antibacterial or antifungal potency of an antibiotic, by the agar streak method (*from* Reilly, Schatz and Waksman, 356).

normally, but began to undergo lysis on the fifth day, large sections of the mycelium disappearing. On the seventh day, only chlamydospores were observed. After 9 days, the fungus completely disappeared, whereas the actinomyces made a normal growth. The antibiotic active against the *Fusarium* was later designated as musarin. It was found to be an optically active acid, of the probable composition of $(C_{35}H_{60}O_{14}N_2)_{72}$, and had an activity of 1:80,000 to 1:100,000 (12).

Leben and Keitt (254) isolated a culture of *Streptomyces* which was antagonistic to 29 phytopathogenic fungi, but not to most bacteria. The culture was grown in corn-steep medium in shake flasks. The culture filtrate was acidified to *p*H 2.5 and the active substance extracted from the precipitate with ethanol. A preparation was obtained which completely inhibited *Venturia inaequalis* in a 1:8,000,000 dilution and *Sclerotinia fructicola* in a 1:11,000,000 dilution. The antibiotic is water-insoluble.

The above antibiotic was designated as antimycin. It was purified by extracting the precipitate produced on acidification of medium to *p*H 2.5 with ethanol. The active material was heat-labile, soluble in various organic solvents and in water at *p*H 9.3. The active substance inhibited the growth of various fungi and of only very few bacteria (255). Several entities were isolated from antimycin preparations and designated as A, B and C. The A was a nitrogenous phenol ($C_{28}H_{40}O_9N_2$). The substance inhibits the respiration of *Saccharomyces cerevisiae,* of cytochrome oxidase and succinic dehydrogenase.

Actinomycetes also exert marked antagonistic effects against species of *Pythium,* as in the case of root rot of sugar cane. Of 3,788 cultures isolated from soil and tested against a parasitic strain of *Pythium,* 896 or 23.6 per cent showed some antagonistic effect upon the fungus, the effect, in some cases, being marked. The occurrence of such antagonistic organisms and the extent of their activities were less pronounced in heavy or infested soils than in light soils (79).

Certain actinomycetes were found (511*a*) to be responsible for the destruction of the mycelium of *Ophiobolus graminis,* the cause of the take-all disease of wheat, in the soil, especially in partly sterilized soils. This parasitizing and antibiotic effect of actinomycetes and of other soil organisms is responsible for the check in the development of *Ophiobolus* in natural soils.

Actinomycetes possess antagonistic properties not only against bacteria and fungi but also against other actinomycetes (275). The more aerobic species are antagonistic to the less aerobic types. Millard (296) believed that he succeeded in controlling potato scab caused by *Streptomyces scabies* by the use of green manures such as grass cuttings. The development of scab on potatoes grown in sterilized soil and inoculated with *S. scabies* was reduced by the simultaneous inoculation of the soil with *Streptomyces praecox,* an obligate saprophyte (299). By increasing the proportion of the latter organism to the pathogen, the degree of scabbing on the test potatoes was reduced from 100 per cent to nil. The sterilized soil provided sufficient nutrients for development of the antagonist, and only a small increase in the control was obtained when grass cuttings were added and sterilized along with the soil.

Sandford (379) was unable, however, to control potato scab by inoculation, with *S. scabies* and *S. praecox,* of either steam-sterilized or natural soil containing different amounts of green plant materials.

These organisms were perfectly compatible on potato dextrose agar, as well as in a steam-sterilized soil medium. The control of scab (299), therefore, was said to have been due, not to the direct action of *S. praecox*, but to certain other undetermined microorganisms favored by the presence of the green manure. *S. scabies* was found (379) to be very sensitive to various products of fungi and bacteria. When grown in close proximity to various bacteria, the acid production of the latter inhibited *S. scabies* to a considerable degree. Its complete inhibition was not due to the acid reaction alone, however, since a certain bacterium which definitely inhibited the growth of this plant pathogen was also isolated from the soil, thus suggesting the possibility that the bacterium may have exerted the antagonistic effect.

Goss (146, 147) observed that the severity of scab is dependent on the amount of *S. scabies* present in the soil. This amount was believed to be controlled by the soil microflora. No evidence was obtained as to whether the effect of the soil flora on *S. scabies* was due to specific organisms. Kieszling (217, 218) isolated two cultures of bacteria which were antagonistic to *S. scabies*. When added to the soil, these bacteria prevented the development of scab on potatoes.

Among the other antagonistic effects of actinomycetes that may prove to be of great economic importance is their action upon nitrogen-fixing bacteria. Konishi and Fukuchi (229) have shown that certain actinomycetes are able to inhibit the growth, on the plate, of root-nodule bacteria; some of the organisms, like *S. flavus*, were particularly inhibiting. In association with actinomycetes, none of the nodule cultures grew readily. In the soil, however, no effect of the actinomyces cultures was observed upon alfalfa bacteria.

The inhibiting effect of actinomycetes upon the growth of *Azotobacter* was first observed by Nikolaieva (323) in 1914. Nickell and Burkholder (322) found in the soil a large number of actinomycetes that exert a marked inhibiting effect upon the growth of *Azotobacter*. It was suggested that antibiosis may be responsible for development of these organisms in the soil.

The antagonistic effects of actinomycetes upon plant pathogenic bacteria has also been well established. Hino (173) isolated several actinomycetes active against *Ps. solanacearum*. *Corynebacterium sepedonicum*, the causative agent of root rot of potato was antagonized by various actinomycetes, some of which produced antibiotic substances and one produced lysis of the bacterium (335). Further studies on this subject were made by McCormack (275). The ability of actinomycetes to produce substances active against bacterial viruses or phages has also been established (198).

In a natural environment, such as the soil, the development of the antagonistic properties among actinomycetes will occur largely under aerobic conditions. In a less well oxidized environment the actinomycetes may themselves be antagonized. A bacterium, like *B. mega-*

TABLE 22: *Classification of antibiotics of actinomycetes:—*

A. Soluble in ether and in other organic solvents:
  I. Pigmented substances:
    1. Orange colored; somewhat soluble in neutral aqueous solution; nitrogen-bearing ring compound, highly toxic, $C_{41}H_{56}O_{11}N_8$; largely active against gram-positive bacteria ..... *Actinomycin*
    2. Yellow pigment, active against both gram-positive and gram-negative bacteria; highly toxic to animals ..... *Xanthomycins A and B*
    3. Compounds related to actinomycin ..... *Actinoflavin*
    4. Red-blue pigment; soluble in aqueous alkaline solution; active against gram-positive bacteria ..... *Litmocidin*
    5. Compounds related to litmocidin ..... *Actinorhodin*
    6. Orange colored; extracted from charcoal adsorbate and from mycelium by ether-alcohol mixture; active largely against *M. tuberculosis* ..... *Nocardin*
    7. Green pigment, active against gram-positive bacteria ..... *Actinomycelin*
  II. Non-pigmented substances:
    1. Organic base; soluble in acidified aqueous solution, inhibits mostly gram-positive bacteria ..... *Proactinomycin*
    2. Largely fungistatic, not bacteriostatic, $C_{27}H_{42}N_2O_7$ ..... *Actidione*
    3. Soluble in water; active against various bacteria and fungi ..... *Mycomycin*
    4. Insoluble in water, present in mycelium; active largely against gram-positive bacteria ..... *Streptocin*
    5. Neutral compound; slightly soluble in water, readily soluble in organic solvents, contains nitrogen (8.6%) and non-ionic chlorine (21.7%); active against various bacteria and rickettsiae ..... *Chloromycetin*
    6. Amphoteric; active against bacteria, rickettsiae and certain viruses *Terramycin*
    7. Heat labile, largely active against fungi ..... *Antimycin*
    8. Heat stable, active against fungi ..... *Fradicin*
    9. An acid; active *in vivo* against the relapsing fever spirochete and enhances the activity of penicillin against the syphilis spirochete ..... *Borrelidin*
B. Insoluble in ether, but soluble in other organic solvents:
  I. Violet-blue pigmented substance ..... *Mycetin*
  II. Colorless, sulphur-containing substance ..... *Sulphactin*
  III. Colorless, nitrogenous body, active against parasitic fungi ..... *Musarin*
C. Soluble in water, insoluble in ether and in other organic solvents:
  I. Bases soluble in aqueous acid solution; removed from charcoal by acid alcohol; active against various gram-positive and gram-negative bacteria.
    1. Little activity against *Bacillus mycoides, Serratia marcescens;* active against Bodenheimer organism and fungi ..... *Streptothricin*
      a. Compounds closely related to streptothricin, but varying in toxicity to animals and showing quantitatively different antibiotic spectra:
        (a) *Streptin*
        (b) *Streptolin*
        (c) *Lavendulin*
        (d) *Actinorubin*
        (e) *Antibiotic* 136
        (f) *Streptothricin* VI and VII
    2. Active against *B. mycoides* and *S. marcescens*, little activity against fungi, no activity against Bodenheimer organism; glycoside (streptidine-streptobiosamine) ..... *Streptomycin complex*

Table 22 (Cont.)

a. Constituents of streptomycin complex:	
(a) Streptidine-streptobiosamine, $C_{21}H_{39}N_7O_{12}$	*Streptomycin*
(b) Mannose derivative of streptomycin	*Mannosidostreptomycin*
(c) Reduced streptomycin	*Dihydrostreptomycin*
b. Streptomycin-like materials:	
(a) *Antibiotic* F, (b) *Streptomycin* II	
3. Active against streptomycin-resistant *M. tuberculosis*	*Neomycin*
4. Basic compounds active against rickettsiae and larger viruses	*Aureomycin*
II. Removed from charcoal by neutral alcohol, soluble in neutral aqueous solutions; narrow antibiotic spectrum against certain gram-positive and gram-negative bacteria	*Grisein*
1. Grisein-like material, still narrower spectrum than grisein, mostly enteric bacteria	*Antibiotic 5310*
D. Proteins and polypeptides:	
I. Colourless preparation, possessing lytic properties against living gram-positive bacteria and dead gram-negative bacteria	*Actinomycetin*
1. Active fraction of actinomycetin	*Actinozyme*
II. Active largely against micrococci; lyses cell membrane	*Actinomyces lysozyme*
III. Combined with orange pigment; largely bacteriostatic against gram-positive bacteria	*Micromonosporin*
E. Incompletely described agents:	
I. Active against the smegma bacillus	*Smegmatis factor*
II. Active against bacteriophages	*Antiphage factors*
III. Active against viruses	*Antivirotics*
F. Agents not produced readily in liquid media; activity obtained only on agar streak:	
I. Little known substances	*Insoluble factors*

therium, may be antagonistic to certain species of actinomycetes but can be antagonized by others. Certain bacteria, like *Ps. fluorescens,* are markedly antagonistic to actinomycetes as a whole, causing their lysis. Numerous fungi are capable of producing antibiotics, such as penicillin and clavacin, which are very effective against actinomycetes.

Production of Antibiotics by Actinomycetes:—Prior to 1940, knowledge of the antibacterial properties of actinomycetes was limited to those of the living organisms. Only two antibiotic substances—one known as actinomycetin and the other as actinomyces lysozyme—were recognized. Both had been isolated only in a crude state. Welsch reported recently (505) in detail upon the antibacterial properties of actinomycetin. The activity of this preparation was expressed in terms of mycolytic units per milliliter of culture filtrate of *S. albus.* A unit was expressed in terms of lysis of a known suspension of heat-killed cells of *E. coli.*

Mycolysis of the heated bacteria by actinomycetin took place at *p*H 3.5 to 11.0 (opt. 7.5-8.5); optimum temperature 38° to 40°. *E. coli* cells killed by chemicals are also dissolved by actinomycetin in a manner similar to the heated cells. The lytic principle is stable at *p*H 5.0 to

9.0; it is thermolabile and is destroyed by ultraviolet radiations. Actinomycetin, as well as the living S. *albus,* has a lytic action upon living gram-positive and upon dead gram-negative bacteria. The bacteriolytic properties of the living S. *albus* and of the actinomycetin preparation upon dead or living cells of bacteria was said to be due to a lytic principle, designated as actinozyme.

Isolation of antibiotics.—The first true antibiotic of actinomycetes was isolated in 1940 from a culture of *Actinomyces* (*Streptomyces*) *antibioticus.* This substance was designated as actinomycin (490). It proved to be highly interesting from a chemical and biological point of view and it affected a large number of bacteria, mostly the gram-positive types. Unfortunately, actinomycin proved to be extremely toxic to the animal body (491) and did not offer, therefore, any chemotherapeutic potentialities.

Later, two other substances, proactinomycin (129) and micromonosporin (468), were isolated. These agents had limited antibacterial spectra and, for one reason or another, they too failed to offer promise as chemotherapeutic agents. Later, other antibiotics were isolated. Attention was concentrated upon the isolation of antibiotics active against gram-negative bacteria, an acid-fast group of bacteria which include the tuberculosis organism. These substances varied greatly in their antibiotic spectra, in their chemical properties, and in their *in vivo* activities.

A number of antibiotics are now known to be produced by actinomycetes, as shown in Table 22. Some of the substances listed are, no doubt, closely related to others or vary from them only in certain minor properties. Some of these substances are produced by different organisms; this is true, for example, of actinomycin, which is formed by a great variety of cultures (465, 505). Some organisms, on the other hand, produce more than one substance; S. *griseus,* for example, produces 2 forms of streptomycin, actidione, and an antibiotic present in the mycelium of the organism, later designated as streptocin (466).

Some of the antibiotics of actinomycetes are active largely on gram-positive bacteria. Others are also active against gram-negative forms. Some, like streptothricin, are active against fungi. Some, like actidione and antimycin, are largely active upon fungi. Some, like neomycin (471) and streptomycin are completely inactive upon fungi. Some are active against trichomonads, as is the case of streptocin. Some are active against rickettsiae and even against certain viruses, including phages (392). These antibiotics also vary greatly in their toxicity to animals. Some, like actinomycin and xanthomycin, are highly toxic. Others, like streptomycin, aureomycin, and chloromycetin, are relatively nontoxic.

The differences in antibacterial action are frequently quantitative rather than qualitative. Streptomycin and streptothricin, for example,

show certain similarities in chemical nature and in their general antibiotic spectra; they differ in their toxicity to animals, in their selective action upon certain bacteria, such as *B. mycoides* and *S. marcescens*, and in the greater action of streptomycin upon *M. tuberculosis hominis*.

Some of the antimicrobial spectra are very narrow, as shown by the so-called antismegmatis factor, which is active only against *M. smegmatis* and certain other mycobacteria (215). On the other hand, streptomycin itself is produced, not only by *S. griseus*, but also by *S. bikiniensis* (194, 195). Streptothricin or similar substances are produced by a large number of organisms. These substances show certain quantitative differences in their action upon different bacteria, in their activity upon fungi, in their toxicity to animals, and in certain chemical characteristics. Usually an organism producing a certain antibiotic is resistant to its antimicrobial action (Table 23).

Table 23: *Inhibition of different actinomycetes by their respective antibiotics* (475):—

Antibiotic	Organism producing it	Activity of preparation per 1 gm	Dilution units per mg, expressed as activity against		
			S. antibioticus	*S. lavendulae*	*S. griseus*
Actinomycin	*S. antibioticus*	100,000*	100	5,000	100
Streptothricin	*S. lavendulae*	100†	1,000	0.4	10
Streptomycin	*S. griseus*	125†	1,000	100	1.2

* *S. lutea* units; crystalline material.
† *E. coli* units; crude preparations.

Methods of isolation and testing.—In a search for antibiotics produced by actinomycetes, several steps are followed, namely,

1. The soil or other natural substrate is plated out on suitable media and the colonies of actinomycetes are picked and transferred to slants.
2. The cultures are tested by the agar-streak method (Fig. 24), using a series of test bacteria. Those that are found to possess the highest or more desirable properties are selected.
3. The selected cultures are grown on suitable media, under stationary and submerged or shaken conditions, and antibiotic spectra of the metabolite solution determined.
4. After suitable media and culture conditions have been established, for a particular organism, it is grown until a large quantity of the metabolic solution is obtained.
5. Methods are now developed for the isolation, concentration, and purification of the antibiotic.
6. The purified antibiotic is now studied for its chemical and physical, as well as its antimicrobial properties, since the antibiotic spectrum of the isolated antibiotic may not correspond to that of the metabolite solution.
7. The antibiotic is now tested for its toxicity to animals and its *in vivo* activity.

Such simple procedures as the agar streak method can be used for screening purposes. The nature of the medium is of great importance, however, as shown in Table 24. Although for most practical purposes, it is sufficient to use ordinary saprophytic bacteria as test organisms, it may become advisable to use in certain cases pathogens. This is true particularly in the search for organisms active against the tuberculosis organism. Williston, Zia-Walrath and Youmans (510) have shown, for example, that for screening of actinomycetes for their antituberculosis activities, the avirulent, rapidly growing strain 607 of *M. tuberculosis* is not suitable; some strains of actinomycetes which inhibit

Table 24: *Distribution of antagonistic properties among actinomycetes* (194):—
Cross-streak method. Numbers reported in per cent of total cultures.

Activity	Zone of inhibition mm	*B. subtilis*	*E. coli*	*M. avium*	*M. phlei*
		Nutrient agar			
Strong	20–35	21	6	6	23
Medium	10–19	46	3	35	35
Weak	1–9	3	13	29	16
None	0	30	78	30	26
		Glucose asparagine agar			
Strong	20–35	15	0	0	6
Medium	10–19	28	6	35	70
Weak	1–9	35	14	8	10
None	0	22	80	57	14

the virulent H37Rv do not inhibit, under the same conditions, strain 607 (Fig. 23*a* and Fig. 23*b*). The nature of the medium is also of great importance, as shown in Table 25.

Isolation of streptothricin and streptomycin.—Streptothricin was the first substance that appeared to show distinct promise as a chemotherapeutic agent, since it was not very toxic to animals, and especially since it was active against gram-negative bacteria. It was obtained (493) from a culture of an organism found to be identical with *Actinomyces* (*Streptomyces*) *lavendulae* that had been isolated in the same laboratory, from the soil, in 1916 (443,460). The name was derived from *Streptothrix*, as the actinomycetes were designated by Ferdinand Cohn in 1875. Other strains of the *S. lavendulae* group were later isolated and found capable of producing streptothricin or closely related antibiotics (179*a*, 204, 215).

Streptothricin is water-soluble and fairly resistant to heat, and is

active against bacteria over a wide *p*H range with an optimum at slight alkalinity. It is also active *in vivo* against various bacteria and fungi. It is not active against viruses. It is resistant to the action of different microorganisms and to enzymes. Unfortunately, it leaves in the animal body a residual toxic effect which precludes its parenteral administration and limits its use to oral or topical applications.

The experience gained in the study of streptothricin proved to be suggestive in planning a search for other antibiotics that would possess similar or even more desirable biological and chemical properties and that would be less toxic to the animal body. After an extensive ex-

Table 25: *Inhibition of growth of virulent human tubercle bacilli by different actinomycetes* (510):—

Actinomyces culture	Inhibition, in millimeters, by agar streak method				
	Medium 1	Medium 2		Medium 5	Medium 6
	H37Rv*	H37Rv	H37RvR†	H37Rv	H37Rv
1	37	25	25	13	32
2	17	15	18	1	21
3	20	0	0	2	17
4	27	9	0	11	27
5	14	11	6	7	15
6	20	16	18	10	17
7	13	3	4	2	14
8	0	0	0	23	0
9	17	20	0	15	15
10	16	18	15	3	18
11	0	0	0	0	0
12	20	3	4	8	12
13	15	15	20	18	14
S. griseus	20	12	0	12	20

* Streptomycin-sensitive strain of *M. tuberculosis*.
† Streptomycin-resistant strain of *M. tuberculosis*.

amination of many cultures of actinomycetes, representing a number of species and strains, two freshly isolated cultures of an organism similar to one isolated from the soil in 1916 and described as *Actinomyces* (*Streptomyces*) *griseus* (460) were obtained and were found to yield an antibiotic which did not possess the toxicity of streptothricin and had an even broader antibacterial spectrum. Since the generic name of this group of actinomycetes had recently been changed from *Actinomyces* to *Streptomyces* (467), the new antibiotic was called *streptomycin*. Different strains of *S. griseus* were later found to vary greatly in their ability to produce streptomycin and in their sensitivity to this antibiotic. The course of growth of this organism, change in the con-

stituents of the medium, and production of streptomycin are illustrated in Tables 26 and 27.

A brief antibiotic spectrum of streptomycin as compared to that of another antibiotic produced by another strain of *S. griseus*, namely grisein, is given in Table 28. The isolation, purification, and practical utilization of streptomycin in clinical medicine have had a most interesting history (390). Of particular interest was the discovery that the

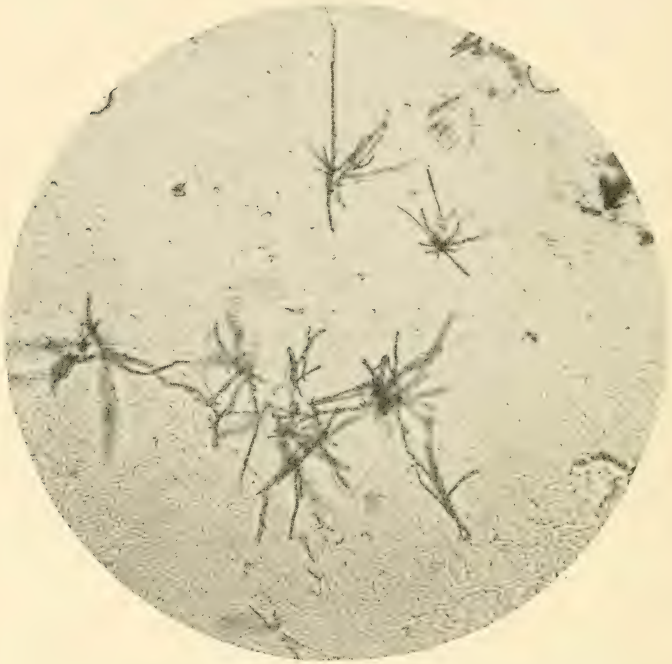

Fig. 25.—Streptomycin-producing strain of *S. griseus*, showing vegetative and aerial mycelium (*from* Waksman and Schatz, 479).

new antibiotic is also active against acid-fast bacteria (394), that it is not very toxic to animals, and that it is active both *in vitro* and *in vivo* against infections caused by various bacteria, including the organism that causes tuberculosis. Before many months had elapsed, streptomycin was tested clinically, and found to be effective against gram-negative bacteria causing a variety of human infections. It was also established that it is effective, not only in experimental tuberculosis, but

in many forms of this disease affecting the human body. The culminating point of these studies was reached in 1946, with the publication by the Committee on Chemotherapy (212) of the reports of one thousand cases in the clinical evaluation of streptomycin and of the first one hundred cases of tuberculosis treated with streptomycin (174).

Streptomycin-producing strains of S. *griseus*.—An organism under

TABLE 26: *Growth and chemical changes produced by S. griseus under submerged conditions* (101):—
Calculated as milligrams in 100 ml culture

	INCUBATION, DAYS					
	0	1	2	3	5	8
*p*H	7.4	7.3	7.6	7.5	8.3	8.9
Mycelium	–	40	510	580	480	380
Streptomycin	–	0	3.7	19.4	23.1	26.7
Glucose	900	880	800	240	60	–
Soluble carbon	1,020	860	700	510	440	460
Lactic acid	29.2	32.8	11.4	1.3	1.6	1.5
Soluble nitrogen	148	130	110	76	73	114
Inorganic phosphorus	11.8	10.8	3.4	0.1	0.2	3.4
Ammonia-nitrogen	6.6	7.0	7.5	6.3	11.5	26.5

the name *Actinomyces griseus* was first described by KRAINSKY in Russia in 1914. In studies of the soil actinomycetes carried out by WAKSMAN and CURTIS in 1915, an organism was isolated from a California soil. This organism appeared to be similar to *A. griseus* Krainsky, so far as could be determined by comparison with the published description of the organism, but not by comparison of the actual cultures. In September 1943, two strains of S. *griseus* were isolated (390)

TABLE 27: *Nitrogen distribution in cultures of S. griseus* (481):—
Per 250-ml portions of broth*

INCUBATION	Nitrogen in mycelium		NH_3-N in broth	NH_2-N in broth	Total N accounted for†
days	*per cent*	*mg*	*mg*	*mg*	*mg*
0	10.0	35	4	35	
4	10.0	35	23	57	115
5	9.7	40	38	70	148
7	10.0	56	56	73	185
10	8.9	62	63	67	193
15	9.6	55	93	79	227
21	7.2	38	95	71	204

* Broth contained per liter 5 gm peptone, 5 gm meat extract, 5 gm glucose and 5 gm NaCl.
† Total nitrogen in original broth 280 mg.

and found to be very similar to the 1915 culture. One of these strains (No. D-1) was isolated from an agar plate streaked with the swabbing of a chicken's throat and the other (No. 18-16) from a heavily manured field soil. The two strains were identical in their morphological and cultural characteristics. They were isolated within two or three days of each other. Although it was believed at first that the second culture could not have arisen from the first, the possibility was not entirely eliminated. Both strains were very potent producers of streptomycin, but they differed in the relative amount of the antibiotic produced un-

TABLE 28: *Antibiotic spectra of streptomycin and grisein* (357):— Units per gram of crude preparations*

	Streptomycin × 1,000	Grisein × 1,000
Bacillus subtilis	125	10 to 30
Bacillus megatherium	100	10 to 20
Bacillus mycoides	20	<.1
Bacillus cereus	30	<.1
Staphylococcus aureus	15	30 to 100
Sarcina lutea	100	0.5
Micrococcus lysodeikticus	150	200 to 300
Escherichia coli W	25	25
Serratia marcescens	25	10
Proteus vulgaris	10	<.1
Pseudomonas fluorescens	2	3
Ps. aeruginosa	1	<.1
Aerobacter aerogenes	10	<.1
Salmonella schottmülleri	15	10
Salmonella aertrycke	3	<.1
Eberthella typhi	25	<.1
Shigella sp.	25	30
Klebsiella pneumoniae	25	5
Mycobacterium phlei	100	<.1

* 1 unit of streptomycin is equivalent to 1 $\mu\gamma$ of pure base. Results are expressed in terms of dilution units against a standard strain of *E. coli*.

der different conditions of culture. No. D-1 was at first the more active strain, but later it declined in activity, whereas No. 18-16 continued to retain its high potency. The latter became the progenitor of all the cultures that are being used at the present time for the industrial production of streptomycin.

When the 1915 isolates of *A. griseus* were later tested for their ability to produce streptomycin and for their sensitivity to the actinophage of *S. griseus* (355), the former were found to produce no streptomycin and to be resistant to the phage.

Upon the irradiation of this culture, KELLNER (p. 73) succeeded in obtaining a mutant which had the capacity of forming typical streptomycin. This mutant was also sensitive to the phage which is active

upon the streptomycin-sensitive strains. The conclusion was reached, therefore, that the streptomycin-producing cultures isolated in 1943 were identical with the 1915 isolate, that the latter has undergone considerable change in culture when grown for 30 years upon synthetic media, and that the 1915 isolate probably possessed the capacity for producing streptomycin, but has lost such capacity upon continuous growth upon artificial media (465*a*).

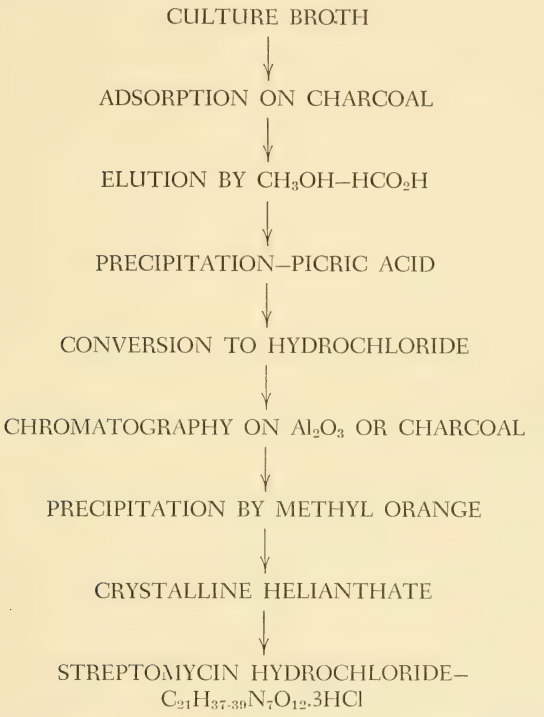

Fig. 26.—Method of isolation of streptomycin from metabolite solution.

Many other strains of *S. griseus* have now been isolated (65, 475) from soils, waters, river muds, animal excreta, dust, and other natural substrates. Only very few of these have been found capable of producing streptomycin, the majority being inactive or producing another antibiotic, such as grisein (357). Ability to form streptomycin may, therefore, be considered as a strain rather than as a species characteristic, in

contradistinction to ability to form penicillin, which is a characteristic property of the *Penicillium notatum—P. chrysogenum* group of fungi as a whole. The streptomycin produced by the active strains of S. *griseus* was found to be made up of several chemical entities, namely, streptomycin and mannosidostreptomycin. Certain other species of *Streptomyces,* such as S. *bikiniensis* (194), appear to produce an antibiotic which is identical with streptomycin. Certain actinomycetes produce a mixture of antibiotics, as streptomycin and streptothricin (428). One of the methods of isolation of streptomycin is presented in Fig. 26.

In order to select the more active streptomycin-producing strains it is necessary to plate out the culture and pick colonies. These show considerable variation in streptomycin production. Several substrains obtained from No. 18-16, such as No. 4 and No. 9, are now largely used, one being more active in certain laboratories, and the other in others. Substrain No. 9 is also more susceptible to the actinophage. Strains which gave an activity of 100 to 200 μg/ml. of streptomycin have been developed to provide strains which give 400 to 500 μg/ml. and even 1,000 μg/ml. Irradiation with ultraviolet light, followed by picking of colonies, has given cultures which yield 600 to 800 μg/ml. The highest producing cultures in one medium are not always the highest in another. A suitable medium must also be selected for inoculation purposes in order to get high yields in the fermenter.

The cultures are kept in a lyophilized state or are first grown on soil then dried, or are continuously transferred on ordinary agar media (65).

Various procedures can be used for the isolation of fresh cultures of S. *griseus* from natural substrates. Ordinary agar media are usually employed, and colonies picked and tested. The S. *griseus* strains can be readily recognized by the pale green to grayish green shade of their aerial mycelium. The agar used for plating purposes may also be enriched with streptomycin, as 25 or 50 mg/ml., to eliminate from the plate the great majority of bacteria and other actinomycetes. To establish the identity of such cultures with streptomycin-producing strains of S. *griseus,* the cultures are treated with S. *griseus* actinophage (474). The following method also offers certain advantages: A suitable agar medium is seeded with living cells of a nonpathogenic strain of *M. tuberculosis.* The diluted soil suspension is added, and the plates are incubated at 28°-30°C. to enable the actinomycetes to develop. This is followed by incubation of the plates at 37°C. to favor development of the *M. tuberculosis.* The antagonistic colonies of the actinomycetes will be surrounded by clear zones, free from the growth of the acid-fast bacteria. By the use of this method, Woodruff and Foster (516) isolated a substance, designated as *streptin,* which was similar in many respects to streptothricin.

Despite the fact that it is possible to isolate large numbers of S. *griseus* strains, only very few of them will be found capable of produc-

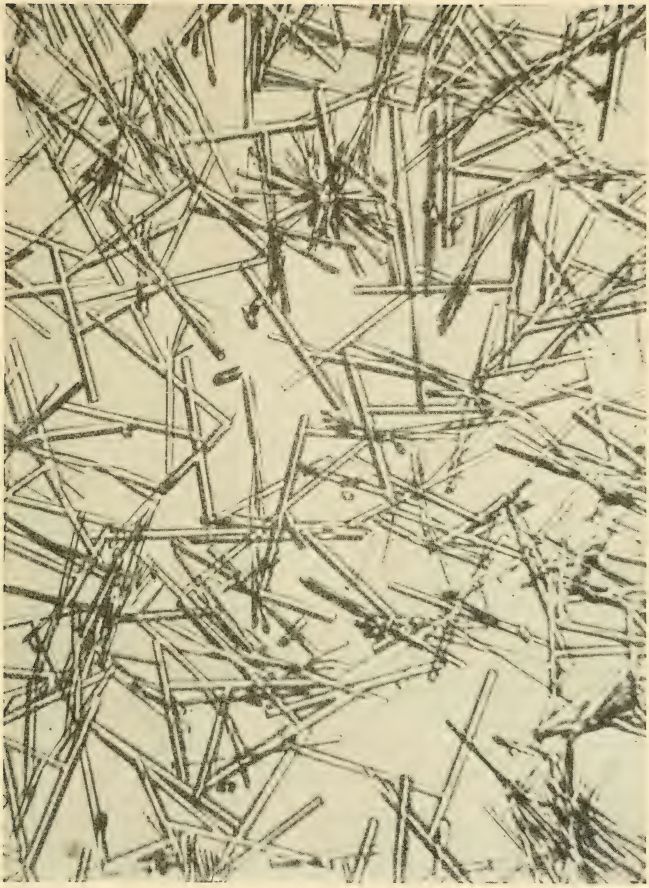

Fig. 27.—Crystals of the calcium chloride double salt of streptomycin (478).

ing streptomycin. Some produce grisein (358), some produce other antibiotics, and some produce no antibiotics at all.

Production of mutants of S. griseus.—When an active streptomycin-producing culture of *S. griseus* is plated out and individual colonies are picked and transferred to agar slants, various inactive strains can be obtained. One such type was found to differ from the mother culture by being free from aerial mycelium (395). This strain undergoes more rapid lysis, especially when grown in submerged culture. It produces

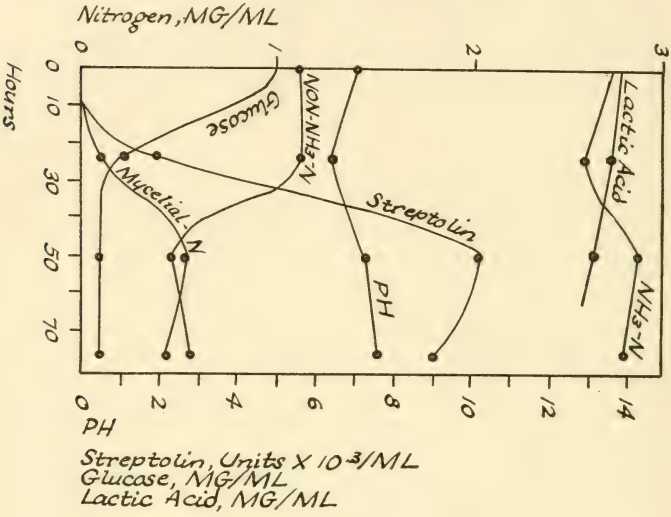

Fig. 28.—Metabolic changes produced in the medium by *Streptomyces* sp. (*from* Rivett and Peterson, 362).

an acid reaction in the medium, and yields a more viscous broth. It is sensitive to the antibiotic action of streptomycin, whereas the mother culture is highly resistant to the action of this antibiotic. The active culture and the inactive variant are similar in many of their cultural characteristics, such as lack of dark pigmentation on organic media, proteolytic action, and hemolytic capacity. By proper cultivation and selection, the inactive asporogenous strain can be made to revert to an active sporulating form which will also produce streptomycin.

Another type of inactive variant or mutant was found to differ from the mother culture in the production of a pink or vinaceous pigmentation in the vegetative growth (475). Some of the cultures of *S. griseus*

are more variable in this respect than others and give rise continuously to strains that appear to be physiologically different, or at least to vary in their quantitative production of streptomycin. The fact that inactive strains are sensitive to streptomycin, whereas the streptomycin-producing cultures are resistant, would tend to bring about the continuous self-purification of streptomycin-producing strains from the nonproducing strains, as long as they are growing under conditions favorable to streptomycin production.

Production of other antibiotics by actinomycetes.—A number of other antibiotics are known to be produced by actinomycetes (482). Some have been crystallized; others have never been obtained in even a concentrated form. Some have wide antibiotic spectra; others act only

TABLE 29: *Antibiotic spectra of streptomycin, streptothricin, and antibiotic 136* (40):—

	Dilution units per milligram		
TEST ORGANISMS	Streptomycin	Streptothricin*	Antibiotic 136
B. subtilis	40,000	6,000	140,000
B. cereus	3,000	50	2,600
S. albus	10,000	1,500	160,000
S. aureus	30,000	7,500	160,000
E. coli	6,000	1,200	17,000
A. aerogenes	5,000	1,200	5,000
Pr. vulgaris	2,000	900	6,000
A. viscosus	8,500	3,750	60,000
Ps. aeruginosa	550	165	1,400
S. marcescens	9,000	1,125	8,500

* This preparation assayed 150 *E. coli* units/mg. On the basis of this comparison, the units for streptomycin and streptothricin should be divided by 8 to make results comparable with standard streptomycin and streptothricin preparations.

against very few organisms. There is also a marked difference in their chemotherapeutic potentialities.

Some of the antibiotic-producing organisms are widely distributed in nature. This is true particularly of such groups as *S. lavendulae* and *S. griseus*. One would expect that some of the strains of these organisms would produce antibiotics which differ in chemical structure and, therefore, in their biological activities. Attention has already been called to the fact that some antibiotics actually obtained from different organisms may either represent a mixture of compounds or a single type compound, which varies, however, in its antibiotic spectrum and in its toxicity to animals. This variation depends upon the strain of organism producing the antibiotic, composition of the medium in which it is produced, and conditions of growth. This can be illustrated by antibiotic 136, which is produced by a strain of *S. lavendulae*, but which differs from streptothricin in its antibiotic spectrum, in toxicity to mice and in the ratio of activity in broth and in agar (TABLE 29).

The fact that certain actinomycetes are capable of producing more than one antibiotic frequently tends to confuse the recognition of the identity of any single constituent. The literature on the antibiotics produced by actinomycetes continues to accumulate rapidly. New agents are being isolated, as in the case of the pigmented substances actinorhodin (46*a*) and actinomycelin (66*a*). New light is being thrown upon the composition and activity of agents previously announced, as in the case of neomycin (179*a*, 469*a*). New fractions are being isolated from older agents, as in the case of antimycin A (101*b*)

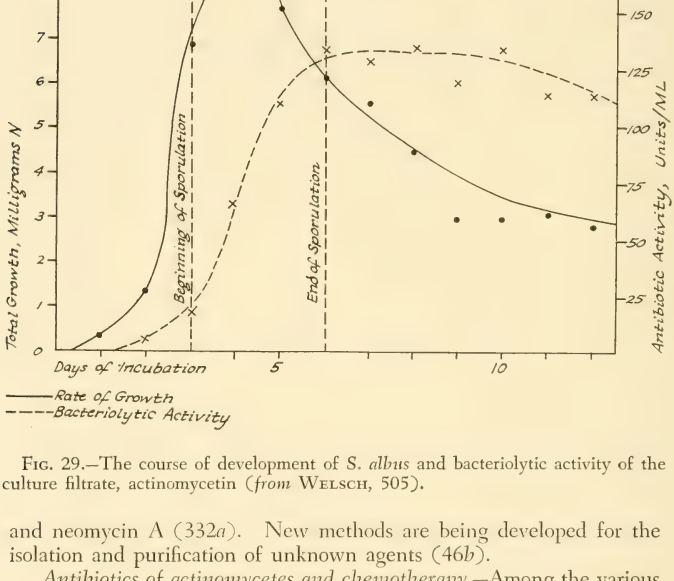

Fig. 29.—The course of development of *S. albus* and bacteriolytic activity of the culture filtrate, actinomycetin (*from* Welsch, 505).

and neomycin A (332*a*). New methods are being developed for the isolation and purification of unknown agents (46*b*).

Antibiotics of actinomycetes and chemotherapy.—Among the various antibiotics produced by actinomycetes, some have already occupied a prominent place as chemotherapeutic agents. It is sufficient to mention streptomycin, aureomycin, and chloromycetin. The most important applications are their use in the treatment of infections caused by gram-negative bacteria, infections by gram-positive bacteria made resistant to penicillin, rickettsial infections, and tuberculosis.

Several volumes have already been devoted to the clinical use of streptomycin. A most extensive literature has accumulated on the use of streptomycin covering nearly 2,000 titles. Comprehensive surveys

of the literature (455) and of the use of this antibiotic in the treatment of numerous infections (456) have already been published. The extensive utilization of streptomycin in tuberculosis has stimulated numerous surveys of the production of antitubercular agents by actinomycetes. Both members of the *Nocardia* (105, 258) and of the *Streptomyces* (193, 471) genera have been investigated in detail.

Among the most important problems that have arisen in connection with the use of streptomycin is the development of resistance among bacteria on contact with this antibiotic (476), and especially the development of streptomycin-dependent strains (300, 331).

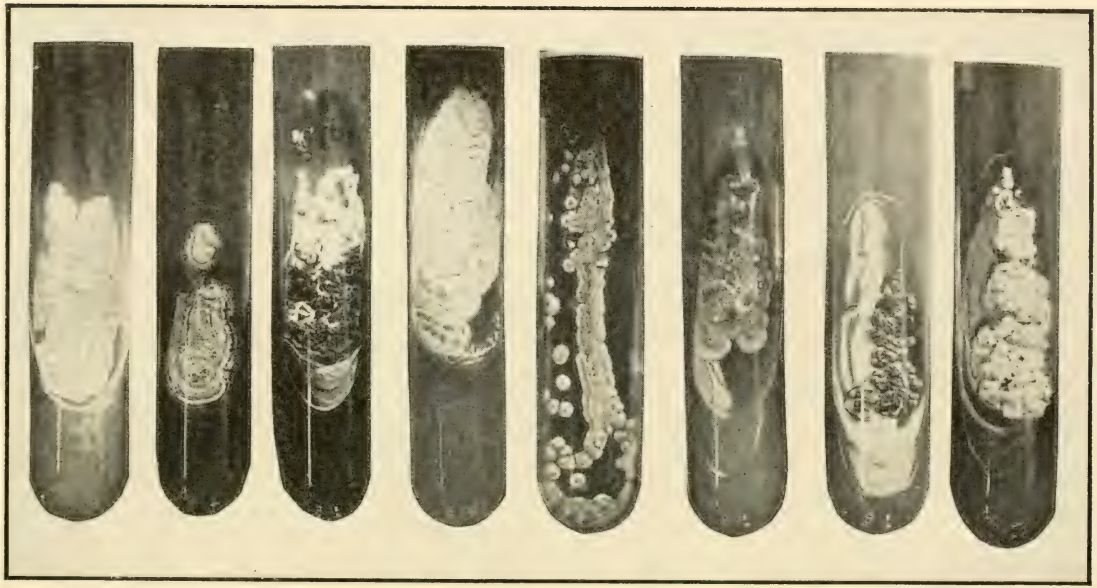

FIG. 30. Typical growth of soil species of *Streptomyces* on synthetic media (*from* WAKSMAN and CURTIS, 460).

Chapter VIII

DISTRIBUTION OF ACTINOMYCETES IN NATURE

Actinomycetes are among the most widely distributed groups of microorganisms in nature. Very few natural substrates are entirely free from them. In some of the substrates, as in soils, in lake water and in lake bottoms, in composts, they lead a normal existence. In other substrates, as in sea water and in dust, they are only in a transitory state.

The ability of actinomycetes to survive for a long time is indicated by the fact that Omelianski isolated an actinomyces (*A. elephantis primigenii*) from the slime of a mammoth's nose. This culture showed no particular properties which would distinguish it from the common actinomycetes found in soils. It must be added, however, that the slime removed from the mammoth, upon its discovery, was kept, without special precautions of preservation, for several months previous to the isolation of the organism (323).

Actinomycetes are found abundantly in all soils throughout the world; they make up, in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil. They also occur on plant residues and upon and in various foodstuffs, such as fruits, vegetables, milk and milk products, and cacao. The open sea is the only important natural habitat where they, like most of the true fungi, are almost entirely absent; whenever their presence has been reported, it was limited to waters close to shore or to waters subjected to land wash, or it was limited to growth upon submerged surfaces, notably piers and other landmarks. Actinomycetes are found in peat bogs, usually in the surface layers where oxygen is present, although occasionally they are also found at greater depths.

Comparatively few types of actinomycetes are known to be capable of causing plant and animal diseases, but both aerobic and anaerobic actinomycetes may be concerned with human and animal infections. Some of these are deep-seated and involve special methods of treatment.

Our knowledge of the occurrence of actinomycetes in nature dates back to the early days of bacteriology. Following the early observations and descriptions of various actinomycetes by F. Cohn and by Bollinger and Harz, Miquel (301), in 1879, in connection with his work on the bacteria of dust over Paris, carefully described certain actinomycetes. The full significance of the nature and importance of these organisms

was not recognized for many years. Macé (278) reported, in 1888, that actinomycetes occur abundantly in water basins. Globig (138), in 1888, and Rossi-Doria (370), in 1891, made a detailed study of their occurrence and activities in different soil types.

In 1900, Beijerinck drew attention to the fact that actinomycetes are widely distributed in nature. They were found not only in the surface layers of the soil but also in the subsoil to considerable depths; at depths of one meter in garden soil and two meters in sandy soils, they sometimes exceeded in numbers the other groups of microorganisms. They were also present in river mud below the river bed. Beijerinck emphasized the fact that actinomycetes represent a group of omnivorous organisms capable of growing not only under conditions favorable to their development but even under certain unfavorable conditions. He even found them to grow in distilled water in ordinary laboratory air. They were unable, however, to fix atmospheric nitrogen.

Nadson (311) also studied in 1900 the occurrence of actinomycetes in nature and their role in natural processes and as geological agents. He isolated several cultures of these organisms from the curative mud of a salt lake, and established their ability to decompose proteins, to produce ammonia and H_2S, and precipitate $CaCO_3$.

Since the work of these pioneers, considerable information has accumulated concerning the abundance of actinomycetes in various natural substrates, as determined by cultural and direct microscopic methods. Although great advance has been made in the appreciation of the role of actinomycetes in many natural processes, no clear picture has been drawn of this function of so large and heterogeneous a group of microorganisms, and the information may still be considered as largely fragmentary. This is due largely to a lack of sufficient knowledge concerning the intermediary metabolism of actinomycetes, their relationship to other microorganisms growing in natural substrates, and their frequent confusion with the bacteria and with the fungi. The last is particularly important, since actinomycetes are capable of bringing about reactions, such as protein decomposition, ammonia formation, nitrate reduction, and cellulose decomposition, which are commonly associated with activities of fungi and bacteria.

Occurrence of Actinomycetes in the Soil:—The soil represents an ideal natural substrate for the development of actinomycetes. It is no wonder, then, that they are found so abundantly there, where they are represented by many genera and species. They are found in both virgin and cultivated soils, in fertile and in unfertile soils, in various regions throughout the world (126, 440, 461). They are particularly abundant in alkaline soils and in soils rich in organic matter. It has even been suggested that their major function in the soils consists in the decomposition of plant and animal residues (76, 484).

Methods of study.—Four methods can be used for determining the presence and abundance of actinomycetes in the soil: *1.* the direct microscopic method of stained soil; *2.* the direct microscopic examination of undisturbed or unstained soils; 3. the contact slide method; and *4.* the plate dilution culture method. Each of these methods has certain distinct advantages and limitations.

Conn (77) was able to demonstrate, by the direct staining of the soil, that the actinomyces mycelium is present abundantly in the soil, especially in soils rich in organic matter. This method does not permit the differentiation between actinomyces spores and certain bacteria. Since the mycelium is not uniformly distributed in the soil, the method does not permit an accurate quantitative evaluation of the abundance of the organism. Furthermore, recognition of individual forms is often limited, since in the process of staining, the mycelium is usually broken up, and both mycelium and spores of various forms will appear similar. The method may, therefore, be limited to the recognition of certain broad groups rather than of specifiic types.

Direct examination of undisturbed natural soils presents certain marked advantages, since it gives a picture both of the relative abundance of this group of organisms in the soil and its distribution through the soil mass. Kubiena and Renn (245) used a vertically illuminated microscope. Actinomycetes were found growing in the soil spaces opening to the surface. Aerial tufts of hyphae in the form of more or less compact colonies with long twisted strands were found to bridge the gulfs between the soil crumbs. When the soil is enriched with organic materials, such as proteins and lignins, the growth of actinomycetes is greatly stimulated.

The contact slide method offers certain advantages over the direct staining method. It permits development of specific organisms upon the slide, and even formation of fruiting bodies, thus making possible the differentiation and recognition of certain broad groups. One is also able to determine by means of this method, not only the gross effects of additions of organic matter and lime to the soil, but also the response in the development of actinomycetes to different types of fertilization and cropping. The relation between pathogenic and saprophytic forms to the root systems of plants can also be studied by the use of the contact slide method. Cholodny (68) demonstrated, for example, that the direct microscopic method gives a rather inaccurate picture of the abundance of actinomycetes, as compared to the contact slide method. The latter has, however, a marked disadvantage, since it gives no idea of the relative abundance of actinomycetes in undisturbed soil.

By the use of the contact slide method, Waksman, Umbreit, and Cordon (487) were able to demonstrate that, in composts kept at different temperatures, the fungi and the bacteria were the first groups of microorganisms to develop at 50° to 65°C.; however, these organisms

were rapidly replaced by an abundant population of actinomycetes. Specific forms could be recognized, by means of this method, and studied in detail.

The plate method has been used most commonly to study the abundance of actinomycetes in the soil and to isolate specific organisms. Synthetic media were found to be highly favorable for the development of these organisms. Some forms grow readily on virtually all common media, whereas others require either certain specific media or special conditions of growth. The actinomyces colonies can easily be distinguished from those of bacteria, a somewhat longer period of incubation usually

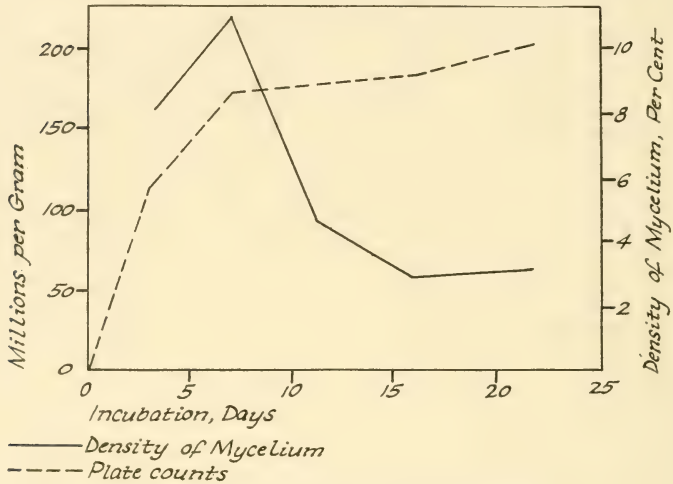

Fig. 31.—Relation between density of vegetative mycelium and plate counts of actinomycetes (*from* Jensen, 192).

being required. This method also has certain limitations, the most important of which is the lack of differentiation between spores and mycelium. A colony of an actinomyces may originate from a single spore or from several spores or from a piece of mycelium. Not all spores are capable of germinating on a given plate and developing into colonies. The numbers thus obtained represent only a minimum content of actinomycetes in a given quantity of soil, or only a fraction of the mass of actinomycetes. This method has been used most extensively, however, for the evaluation of the actinomyces population of the soil.

Abundance of actinomycetes.—Hiltner and Störmer (172), in 1902, made the first comprehensive survey of the abundance of actinomycetes, as compared to that of bacteria, in the soil. In the spring of the

year, 20 per cent of all the colonies developing upon an ordinary agar plate, when various soils were plated out in accordance with accepted bacteriological procedures, consisted of actinomycetes. In the fall, the number of actinomycetes increased to 30 per cent of the total microbial population developing on the plate. This increase was believed to be due to the greater amounts of fresh plant residues becoming available at that time of year. In the winter, there was a drop in the relative num-

TABLE 30: *Numbers of bacteria and actinomycetes in the soil developing on albumen agar* (460):—
Per 1 gm of soil

	Soil type	Bacteria	Actinomycetes	
		Numbers	Numbers	Per cent
1	New Jersey Sassafras garden	5,300,000	900,000	14.5
2	New Jersey orchard	4,800,000	700,000	13.4
3	New Jersey clay meadow	8,100,000	550,000	6.3
4	New Jersey Sassafras forest	610,000	110,000	15.3
5	Iowa Carrington loam	1,764,000	236,000	11.8
6	Jamesburg Cranberry soil	204,500	7,500	3.5
7	Louisiana sandy loam	8,300,000	1,700,000	17.0
8	California fertilized soil	3,570,000	630,000	15.0
9	California unfertilized soil	580,000	330,000	36.3
10	California upland	2,220,000	1,238,000	35.8
11	California adobe	3,620,000	800,000	22.0
12	California sandy loam	6,010,000	1,430,000	19.2
13	Oregon adobe	13,100,000	2,400,000	15.4
14	Oregon white land	3,400,000	300,000	8.1
15	Porto Rico clay loam	2,140,000	960,000	31.0
16	North Dakota wheat soil	2,067,000	933,000	31.1
17	North Dakota flax soil	1,737,000	263,000	13.2
18	Hawaiian pineapple soil	4,334,000	666,000	13.3
19	Alaska soil	6,034,000	1,566,000	20.6
20	Texas Lufkin fine sandy soil	2,126,000	574,000	21.3
21	Colorado alfalfa soil	2,440,000	1,560,000	39.0
22	Maine Aroostook potato soil	4,650,000	250,000	5.1
23	Maine dark Aroostook infected	15,900,000	2,200,000	12.2
24	Alberta grass soil	1,110,000	760,000	40.6
25	Alberta garden soil	2,000,000	1,700,000	46.0
	Average	4,245,000	870,500	17.0

ber of actinomycetes to 13 per cent, believed to be due to the effect of frost. When the soil was treated with stable manure, there was a marked increase in the total and relative numbers of these organisms.

FISHER (122) reported a much smaller number of actinomycetes in various soils, seldom exceeding 15 per cent of the total number of organisms developing on the plate. This may have been due to different media used in the evaluation of the abundance of these organisms in the soil.

TABLE 31: *Distribution of actinomycetes in various soils* (460):—

SOIL TYPE	*S. coelicolor*	*S. novaecaesareae*	*S. purpeochromogenus*	*S. viridochromogenes*	*S. exfoliatus*	*S. diastaticus*	*S. albus*	*S. alboatrus*	*S. reticuli*	*S. citreus*	*S. griseus*	*S. alboflavus*	*S. verne*	*S. albosporeus*	*S. californicus*	*S. lipmanii*	*S. rutgersensis*	*S. aureus*	*S. fradiae*	*S. roseochromogenus*	*S. lavendulae*	*S. bobiliae*	*S. griseolus*	*S. flavotirens*	*S. erythreus*	*S. flaveolus*
New Jersey garden soil				+			+			+						+	+	+		+		+	+			
New Jersey orchard soil							+			+		+				+	+	+			+					
New Jersey forest soil																		+								
New Jersey meadow soil							+					+					+	+								
Iowa loam	+									+						+		+								
Louisiana loam					+											+	+	+								
California fertilized				+												+					+					+
California unfertilized				+											+	+									+	
California adobe	+		+		+		+	+	+		+					+			+			+				
California upland		+			+				+			+	+	+												
California loam						+									+											
Oregon adobe				+			+			+	+					+		+					+			
Oregon white land				+																	+					
Porto Rico loam				+																						
North Dakota wheat soil																	+	+								
North Dakota flax soil																+		+								
Hawaiian soil																+		+							+	
Alaska soil																+	+	+								
Texas soil				+							+					+	+	+								
Colorado soil							+										+	+		+			+			

In general, a close correlation has been obtained between the abundance of actinomycetes in the soil and the amount and extent of decomposition of available organic matter. Heinze (167) spoke of actinomycetes as playing an essential role in the soil "fermentation" processes and in humus formation. Macé (279) demonstrated that actinomycetes are capable of decomposing proteins to a very marked extent, thus pointing to their importance in the decomposition of plant residues in the soil.

Fousek (125) made a comprehensive study of the ability of actinomycetes to break down plant and animal residues, and came to the conclusion that these organisms play an important role in decomposition processes. He obtained considerable variations among the different soils: loam soils contained much larger numbers than sandy soils, uncultivated soils more than cultivated soils, and the numbers were higher in fall than in spring. This was also explained by the increase, in the fall, of fresh undecomposed plant residues which serve as food for the actinomycetes. These organisms were also abundant in forest soils (24 to 27 per cent), pointing to their important role as agents of decomposition and humus formation.

According to Conn (77), soils contain much larger total numbers of actinomycetes, namely, 12 to 14 millions per gram, than those reported by Hiltner and Störmer, namely, 2.5 millions. Actinomycetes were found to be particularly abundant in old sod soils, where they made up nearly 40 per cent of the total soil microflora, as compared to about 20 per cent of the corresponding microflora in cultivated soil. Conn found that the addition of grass roots to soil stimulated the development of actinomycetes.

It may be reasonably concluded that the exact number of actinomycetes in any one soil depends, not only upon the nature of the soil and upon its treatment, but also upon the medium that is used for making the plates and upon the conditions of incubation. This explains why Hiltner and Störmer and Conn reported the numbers of actinomycetes in the soil to be greatly in excess of one million per gram of soil, whereas Krainsky (230), who used for plating purposes a very special medium, not favorable to the development of large numbers of actinomycetes, found only 20,800 organisms per gram.

By the use of simple synthetic media, such as nitrate-sucrose agar or egg-albumen agar it was found (Table 30) that the numbers of actinomycetes varied in 25 soils from 7,500 per gram, for an acid cranberry soil, to 2,400,000 for an Oregon adobe soil; these numbers represented 3.5 and 15.4 per cent of the total microbial population developing on the plate respectively. Soils rich in organic matter contained a microbial population which was made up of 39 to 46 per cent actinomycetes.

There was a marked difference in the nature of actinomyces types found in the different soils. Some of the species were very abundant and were found in several soils, whereas others were only of limited oc-

currence and were detected only seldom in one or two soils (Table 31). With an increase in depth of soil, there was a remarkable increase in the proportion of actinomycetes, as compared with the total soil microbiological population. Based on an average of three soils, the relative number of actinomycetes increased from 9.2 per cent in the surface layer of 2 cm to 65.6 per cent at a depth of 75 cm, although the total number of organisms decreased from 743,000 to 240,000 per gram between these two depths.

Jensen (187) made a detailed study of the abundance of actinomycetes in various Danish soils. The total numbers were found to range

Table 32: *Distribution of microorganisms in different soils from Bikini and Rongelap Islands* (194):—
Total numbers per gram of soil

Organic matter per cent	*p*H	Bacteria	Fungi	Actinomycetes
		Bikini soils		
1.02	9.2	100	100	2,900
0.60	9.2	100		4,700
0.60	9.2		200	3,200
1.40	8.7	3,000	500	17,000
0.80	8.7	1,000		58,000
0.52	8.7	1,000		36,000
		Rongelap soils		
4.40	8.3	160,000		600,000
1.84	8.4	100,000		800,000
0.32	8.4	3,000	1,000	10,000
0.32	8.0	1,000		191,000
0.45	8.0			4,000,000

between a few thousand to 13 million per gram. The acid soils, of a *p*H less than 5.0, had only very few actinomycetes. The numbers increased with a decrease in acidity, largest numbers occurring in soils of *p*H 6.8-8.0. The relative abundance of actinomycetes as compared to the total population varied from 0, for acid peat soils, to 37 per cent. Some of the species were very common and were found in many soils, whereas others were observed in only very few instances. In a study of Bikini soils, which are very alkaline in reaction, Johnstone (194) found that the microbiological population is made up largely of actinomycetes (Table 32).

Influence of soil treatment upon abundance of actinomycetes.— Treatments of soil, especially the use of fertilizers which result in changes in soil reaction and the use of the organic manures and cover crops, greatly influence the abundance of actinomycetes in the soil (Table 33).

By means of the contact-slide method, JENSEN demonstrated (192) that the development of vegetative mycelium of actinomycetes in the soil is favored by a low moisture content and by an increase in temperature from 5° to 28°C. Growth is very scanty at 5°C., whereas at 37°C., there is no further stimulation of vegetative growth. The addition of $CaCO_3$ to an acid soil results in a marked increase in actinomycetes (187) as shown in TABLE 34.

TABLE 33: *Influence of 1 per cent dried blood upon the microbiological population of the soil* (484):—
Numbers in thousands per gram of soil

		Fungi		Bacteria		Actinomycetes	
SOIL TREATMENT*	*p*H	Start	After 12 days	Start	After 12 days	Start	After 12 days
Manure	5.5	87	2,080	4,700	190,900	1,800	190,900
Manure + lime	6.7	20	73	6,000	367,000	3,360	6,000
None	5.1	116	1,438	2,600	471,700	1,260	2,200
Lime alone	6.5	20	125	5,000	352,200	2,760	500
$NaNO_3$ fertilizer	5.8	73	1,872	6,500	183,500	1,500	128,700
$(NH_4)_2SO_4$ fertilizer + lime	6.0	26	311	5,700	65,200	2,700	42,700

* All soils cropped and treated for 15 years.

In a study of the effect of partial sterilization of soil, it was found (483) that actinomycetes were adversely affected by high concentrations of toluol but were more tolerant of low toluol concentrations than were the bacteria. The relative abundance of the actinomycetes increased when the bacteria decreased, whereas the relative abundance of actinomycetes decreased with an abrupt increase in the numbers of bacteria. In general, in response to soil treatment, the actinomycetes were not so subject to rapid changes in numbers as were the bacteria.

TABLE 34: *Influence of addition of $CaCO_3$ on the numbers of actinomycetes in acid soils* (187):—
Millions per gram of soil

Nature of soil	Incubation days	No $CaCO_3$			$CaCO_3$ added		
		*p*H	No.	per cent	*p*H	No.	per cent
Heath soil	Start	3.85	0	0	–	0	–
— —	15	–	0	0	7.52	18.3	22
— —	45	3.65	0	0	7.47	84.9	21
— —	75	3.90	0	0	7.59	122.9	30
Field soil	Start	5.92	2.4	32	–	2.4	32
— —	15	6.04	2.7	24	7.72	3.0	46
— —	90	5.78	2.9	36	7.62	3.5	20

After moistening of the air-dried soil, the ratio of actinomycetes to the total numbers of microorganisms, determined by plate counts, declined as the bacterial numbers increased. This was followed by an increase in the ratio of actinomycetes due to a decrease in the numbers of bacteria, until a nearly constant ratio of 20 to 25 per cent of numbers of actinomycetes to total colonies was established. When soil was heated to 65° for 1 hour, the ratio of actinomycetes to total numbers of colonies on the agar plate changed from an initial 30 per cent to 15 per cent. After 2 weeks, only 3 per cent of the colonies were those of actinomycetes. This was followed by an increase in the relative numbers of actinomycetes. However, even 4 months after treatment the ratio of actinomycetes to total numbers was only one-fourth the ratio before treatment.

Specific nature of soil actinomycetes.—Of the four genera of the *Actinomycetales,* three are represented abundantly in the soil. A large number of species belonging to all these genera have been isolated and described. The aerial mycelium producing *Streptomyces* species are the most common; these are followed by the *Nocardia* types. The *Micromonospora* group is also well represented in the soil; several forms have been isolated and described, but because of their slower growth on the common media under the usual conditions of cultivation, only few species have so far been recognized. They are particularly abundant in high-temperature composts and in lake bottoms. Thermophilic actinomycetes are particularly abundant in soils receiving stable manures (459).

The anaerobic group, that is, the members of the genus *Actinomyces,* have so far not been reported isolated from the soil. Although they are brought into the soil in the diseased bodies of animals, it is not known to what extent they are able to survive there. It is recognized, for example, that certain pasture lands are apparently favorable to infection with lumpy-jaw of grazing cattle. This would seem to point to the presence of *A. bovis* in the soil, at least under certain conditions. Because of the need for special methods of isolating and cultivating these organisms, no attempt has ever been made to isolate them directly from the soil. During a survey of the microbial population of peat bogs (473), an organism belonging to the anaerobic group was isolated from a Florida peat. It grew only under anaerobic conditions and at a temperature above 40°C. It was definitely a species of *Actinomyces,* but, unfortunately, no further study was made of this culture.

Many actinomycetes have been isolated from the soil and have been described as distinct species. Certain others have been recognized, but have been described not as species but rather as group species, since the overlapping among various strains makes it rather difficult to establish species types. Without a sufficiently clear differentiation, on the one hand, between certain actinomyces-like organisms and true bacteria, and, on the other, between different genera, especially *Nocardia* and

Streptomyces, it often becomes difficult to say with assurance whether a certain culture represents a known species or is merely a variable strain. The soil harbors many microorganisms which resemble nocardias and which show a close relationship to the mycobacteria or even to the corynebacteria. This makes the recognition of well-defined types of actinomycetes particularly difficult (425). The difficulty is further complicated by the great heterogeneity and even pleomorphism of the

TABLE 35: *A list of typical actinomycetes occurring in soils and in composts:—*

S. acidophilus (Jensen)	*S. halstedii* (Waksman and Curtis)
S. albus (Rossi-Doria em. Krainsky)	*S. hygroscopicus* (Jensen)
S. antibioticus (Waksman & Woodruff)	*S. lavendulae* (Waksman & Curtis)
S. aureus (Waksman and Curtis)	*S. melanocyclus* (Krainsky)
S. bobiliae (Waksman and Curtis)	*S. microflavus* (Krainsky)
S. californicus (Waksman and Curtis)	*S. olivaceus* (Waksman)
S. cellulosae (Krainsky)	*S. olivochromogenus* (Waksman)
S. coelicolor (Muller)	*S. parvus* (Krainsky)
S. cretaceus (Krüger)	*S. phaeochromogenus* (Conn)
S. diastatochromogenes (Krainsky)	*S. reticuli* (Waksman and Curtis)
S. erythrochromogenes (Krainsky)	*S. roseochromogenes* (Krainsky em. Jensen)
S. exfoliatus (Waksman and Curtis)	*S. ruber* (Krainsky)
S. flavus (Krainsky em. W & C)	*S. rutgersensis* (Waksman & Curtis)
S. fradiae (Waksman and Curtis)	*S. scabies* (Thaxter)
S. fulvissimus (Jensen)	*S. verne* (Waksman and Curtis)
S. griseoflavus (Krainsky)	*S. verticillatus* (Kriss)
S. griseolus (Waksman)	*S. violaceus* (Gasperini)
S. griseus (Krainsky em. W & C)	*S. viridochromogenes* (Krainsky em. W & C)
N. actinomorphus (Gray and Thornton)	*N. gardneri* (Waksman)
N. agrestis (Gray and Thornton em. Jensen)	*N. mesentericus* (Orla-Jensen em. Jensen)
N. alvi (Peklo)	*N. minimus* (Jensen)
N. corrallina (Hefferan em. Jensen)	*N. opacus* (den Dooren de Jong em. Jensen)
N. elaeagnii (Boberg)	*N. paraffinae* (Jensen)
N. erythropolis (Gray and Thornton em. Jensen)	*N. polychromogenes* (Vallée em. Orskov)
N. flavescens (Jensen)	*N. salmonicolor* (den Dooren de Jong em. Jensen)
M. chalcea (Foulerton) Jensen	*M. fusca* (Jensen)
M. coerulea (Jensen)	*M. parva* (Jensen)

nocardias. Because of the often questionable recognition of organisms belonging to the genus *Nocardia* and because only few *Micromonospora* species have so far been isolated from the soil, there is a general belief that the genus *Streptomyces* should be recognized as representing the true and dominant soil types of actinomycetes. This merits certain justification, especially in view of the fact that when a species of *Streptomyces* loses the capacity of producing aerial mycelium it may well be considered as a typical species of *Nocardia* (395).

A list of typical soil actinomycetes is given in TABLE 35. These forms may be regarded as established types of soil organisms. In addition to the forms here listed, many others have been isolated and de-

scribed. Because of insufficient recognition of their relative abundance and possible importance, most of these can be omitted from consideration at the present time. A detailed listing of such types is found in the latest addition of Bergey's Manual.

Occurrence of Actinomycetes in Manures and Composts:—Next to the soil, composts of stable manures and of other plant residues present the most extensive source of actinomycetes. Under certain conditions, as under proper aeration, rapid decomposition of the organic constituents of the manure results, and the temperature rises to 60° C. and above. This is accompanied by such abundant development of actinomycetes as to virtually replace the rest of the microbiological population and become visible to the naked eye in the form of white or gray masses all through the upper layers of the manure pile. These organisms are largely thermophilic in nature. Hence, a consideration of the actinomyces population of manures and composts must give first consideration to thermophilic forms. A detailed discussion of these organisms has already been presented (p. 97).

In a recent study of the occurrence of thermophilic actinomycetes in high-temperature composts (459, 487), two general groups, similar to those reported by earlier investigators were observed. These may be said to be represented by members of the genus *Streptomyces* and by various species of *Micromonospora*.

Soils treated with stable manures also contain large numbers of these organisms. Although the thermophilic actinomycetes grow at 50° and 65°C., many of them can also grow readily at 28°C. They are, therefore, not obligate but facultative thermophiles. When cultures of thermophilic actinomycetes grown on agar media were added to the soil and kept at room temperature, they died out rapidly. When they were introduced with the thermophilic composts, however, they survived in the soil. When the soil was kept at 28°C., there was no multiplication of the thermophilic actinomycetes. However, composts of horse manure kept at 50° and 65° developed an extensive and highly characteristic population of thermophilic fungi and actinomycetes, as shown in Table 36. The addition of organic matter to the soil has also a marked favorable effect upon the abundance of thermophilic actinomycetes.

Occurrence of Actinomycetes in Water Basins:—Although waters in general contain very few actinomycetes, fresh-water lakes and certain river waters may contain a highly characteristic population made up partly of actinomycetes. Macé was the first to establish the fact that actinomycetes are found in water basins. Nadson extended this work and demonstrated that actinomycetes are found abundantly in lake muds. No attention was paid to the fact, however, that these organisms represent only certain special types which are characteristic of this substrate.

It has been recently established that the actinomycetes found in water basins, especially in lake bottoms, are largely members of the genus *Micromonospora.*

Erikson (113) isolated from lake mud, and in one instance from lake water, ten strains of *Micromonospora chalcea.* These forms were found capable of growing on a large variety of more or less resistant organic compounds and were especially active in the decomposition of chitin, cellulose, pentosans, glucosides, and, to a lesser degree, of lignin. It was suggested that the *Micromonospora* types, because of the resist-

Table 36: *Influence of temperature upon the development of microorganisms in manure composts* (459):—

Per gram of moist compost

Temperature of incubation	Period of incubation	Bacteria	Actinomycetes	Fungi
°C.	*days*	*millions*	*millions*	*thousands*
Start	0	1,600	0.2	200
28	2	14,000*	.....	0
	8	175*	.....	.....
	21	85*	.....	11,000
	39	50*	.....	600
50	2	100*	.....	.....
	5	850	150	.....
	8	1,000	1,000	.....
	21	Few	14	2,000
	39	0	6.4	1,000
65	2	100*	.....	0
	5	.....	2	0
	8	.....	106	0
	21	.....	2.5	0
	39	.....	7.6	0
75	8	3.5	0	0
	21	2.0	0	0

* Including actinomycetes.

ance of their spores, may be better adapted to life under aquatic conditions than are other actinomycetes with aerially borne spores.

Umbreit and McCoy (433) found that 10 to 20 per cent of the total microbiological population found in the water of the lakes of the northern highland region of Wisconsin comprised species of the genus *Micromonospora.* In some cases these numbers were as high as 40 per cent, as in the surface water of Trout Lake, with a total of 250 organisms per milliliter. The water of Crystal Lake contained 3,600 organisms per milliliter, of which 16 per cent were made up of members of the genus *Micromonospora.* Even larger numbers of these organisms were found in the bottoms of the lakes. The deposit of Lake Mendota,

while still covered with ice, contained 12,500 cells of *Micromonospora* per milliliter of wet mud, or 16 per cent of the total microbiological population. After the ice had left, the numbers increased to 100,000 or 45 per cent; they reached 120,000 to 250,000 or 11 to 22 per cent in June. This relationship was found to hold true, not only for the shallow, but also for the deeper bottom deposits, where the proportion of members of the *Micromonospora* group to the total population might even be greater. The microbiological population of the edge of the lake, the beach area, tended to approach that of normal soil, with various other actinomycetes as well as fungi making their appearances. The genus *Micromonospora* thus appears to form a group of microorganisms which are indigenous inhabitants of lake waters and lake water deposits.

Thaysen (417) found as many as 8,400 to 609,000 actinomycetes per gram of submerged river mud, 120 to 1,100 meters below the tidal line. There was a definite parallelism between the strength of the earthy odor in the mud and in the water and the abundance of actinomycetes. These organisms were believed to be concerned with the destruction of the submerged reeds, since the bases of the reeds yielded the greatest numbers of actinomycetes.

Putilina (351) made a detailed study of the abundance of actinomycetes in the waters and sediments of the Don basin, for a distance of 120 km. The numbers of these organisms in the bottom material were 10 to 1,000 times greater than those in the water. These actinomycetes were believed to be responsible for the undesirable odors imparted to the Don waters. Similar odors were produced when the isolated cultures were artificially cultivated.

Egorova and Issatchenko (102, 182) also reported an extensive population of actinomycetes in river bottom deposits, the numbers varying from 36,585 to more than a million per gram of dry material. Large numbers were found even at depths of 20 cm. Only few actinomycetes (100 per milliliter) were found in the water itself. These organisms were largely of the aerobic, sporulating types. When some of the water and a layer of bottom material were placed in cylinders, sterilized, and inoculated with pure cultures of actinomycetes, excellent growth was obtained in a short time. This suggested the aqueous existence of these organisms. The earthy smell and the unpleasant flavor of the Moskau river water was ascribed to the multiplication of these actinomycetes in the bottom muds, especially during the summer and fall months, when extensive multiplication of these organisms occurs at the expense of the abundant plant remains.

Actinomycetes occur very extensively in peat bogs, especially in the sedge and reed peats, which are less acid in reaction. Their presence in these habitats is limited largely to the upper aerated or partly aerated layers, as shown in Table 37. When the peat bog is drained, actinomycetes develop extensively.

The occurrence of actinomycetes on submerged surfaces in sea

waters as well as in marine bottom deposits has also been reported (522). Rubentschik (371) isolated from the salt liman of the Black sea an organism, *A. melanogenes,* which decomposed cellulose. ZoBell, Grant, and Haas (521) included species of *Streptomyces* and *Micromonospora* among the organisms which attack aliphatic, aromatic, naphthenic, and olefinic hydrocarbons under aerobic conditions. These organisms were said to be widely distributed in sea water and in marine mud.

Occurrence of Actinomycetes in Dust and on Exposed Surfaces of Plants:—Actinomycetes, especially forms producing aerial mycelium, are abundant in the dust of the atmosphere and are found on all exposed surfaces. They are largely derived from the soil, as is indicated by their similarity to the soil-inhabiting forms. As has been pointed out,

Table 37: *Numbers of microorganisms in an undrained peat bog in Florida* (473):—
Numbers in 1 gm of moist peat

Depth of peat	Moisture	Aerobic bacteria	Actinomycetes	Fungi	Anaerobic bacteria
cm	*per cent*				
2–20	80.1	890,000	370,000	20,000	120,000
22.5	82.9	960,000	290,000	10,000	180,000
45	85.3	410,000	100,000	7,000	180,000
75	84.0	18,000	13,000	330	16,000
120	85.4	30,000	330	0	75,000
165	87.4	235,000	3,330	0	380,000

the first observations on the occurrence of actinomycetes in the dust were made by Miquel, in 1883, in his studies on the microorganisms of the atmosphere. Since then, many similar reports have been made. The universal presence of these organisms and their ability to thrive on traces of organic materials often led to generalizations that seemed hardly justified. It is sufficient to mention, for example, the organism designated by Beijerinck as *B. oligocarbophilus,* a form later (251) shown to be an actinomyces. As the name indicates, it was believed capable of obtaining its carbon and energy from everywhere, including the dust of the atmosphere. Even less justified is the assertion of Emerson (104) that actinomycetes may be looked upon as organisms capable of fixing atmospheric nitrogen. This assertion was based upon the development of actinomyces colonies when soil was plated out on nitrogen-free agar media.

The assertion (262*a*) that living organisms resembling actinomycetes were discovered in Pre-Cambrian rock and in Pliocene rock has never been confirmed. The chances are that they were derived from the

air which had access to the rock, either in a native state or during the handling in preparing the plates.

Occurrence of Actinomycetes in Foodstuffs:—Actinomycetes, chiefly forms producing aerial mycelium, are present abundantly on the surface of various fresh and dried food materials. Whenever actinomycetes have an opportunity to develop upon such materials, especially during storage, they may cause considerable damage through formation of typical earthy odors that impart to the foodstuffs undesirable flavors. It is particularly interesting to note that actinomycetes are capable of developing upon foodstuffs under conditions not very favorable to molds and bacteria, namely, at fairly high temperatures and at low moisture contents. Whenever foodstuffs are exposed, under aerobic conditions, at a moisture content which is suboptimum for development of other spoilage-producing microorganisms and at temperatures which are too high for such development, they are subject to attack by actinomycetes.

Only very few food materials have received attention from this point of view. It is sufficient to mention, for example, the cacao bean. Several cultures of *Streptomyces* were isolated from beans received from Nigeria (55) and from cacao produced in the Dominican Republic (69). The mustiness of the beans was ascribed to several species described as *A. albus* and as *A. cacaoi*. Milk is also subject occasionally to spoilage by actinomycetes, but this problem, as well, has not been studied systematically (117).

Occurrence of Actinomycetes in Animal Systems:—Aside from the strictly pathogenic forms, actinomycetes are frequently found in various organs of animals. An organism described as *Streptomyces rhodnii* has been isolated from the insect *Rhodnius prolixus* reared in the laboratory (45). This organism is not transmitted through the egg but is taken up by the young nymph from the environment, such as the contaminated surface of the egg; more often it is transmitted to the insect by the dry excreta of other members of the species. The insect has been reared free from the actinomyces by sterilizing the surface of the egg and feeding the adult with suitable precautions. These sterile insects grow and moult normally only for a certain period. Very few of the insects that have been reared free from the actinomyces become adult, and those few are usually incapable of reproduction. Normal growth, moulting, and egg production by these insects are resumed when they are reinoculated with the actinomyces.

Chapter IX

DECOMPOSITION OF PLANT AND ANIMAL RESIDUES

The ability of actinomycetes to decompose various organic chemical compounds suggests the probable importance of these organisms in the destruction of complex plant and animal materials in nature, especially in soils, water basins, and composts. Most of the investigations upon which these results are based have been carried out by means of pure cultures. The elucidation of the role played by actinomycetes in the decomposition of complex organic compounds under natural conditions must, therefore, be considered as somewhat arbitrary. Generalizations of the function of certain members of a complex microbiological population on the basis of results obtained with single organisms and pure chemical compounds have often been faulty. This is true particularly of interpolations of decomposition processes of complex organic materials in complex substrates on the basis of results obtained by the use of pure cultures of actinomycetes acting upon pure chemical compounds under artificial conditions, especially in agar or liquid media. The above considerations are further complicated by the fact that the organisms used for decomposition studies are indefinitely or even incorrectly described. Most of the organisms have been designated by the generic name *Actinomyces,* thus making it impossible to determine the true nature of the organism involved. Because of the extreme variation in the growth conditions of different actinomycetes, results obtained by the use of liquid or agar media in which certain unknown cultures have grown can hardly be applied to the activities of an extensive population of actinomycetes growing in a complex substrate in the presence of many bacteria and fungi, where the organisms are subject to a variety of associative and antagonistic effects.

Decomposition of Pure Organic Compounds:—In a comparative study of the decomposition of amino acids by different microorganisms, certain cultures of actinomycetes were found (472) to attack glycine and alanine more effectively than did the fungi employed. More abundant growth of the actinomycetes was accompanied by greater ammonia production, as shown previously (p. 81). Glutamic acid, however, allowed better growth of the fungi and poorer growth of the bacterium, the actinomyces growth being intermediate. The amount of ammonia liberated was alike for all three organisms.

A more detailed study of the utilization of glycine by different microorganisms (p. 81) brought out some very interesting facts. In the presence of glucose, the amount of growth made by the fungus *Trichoderma* was much greater than that made by the *Streptomyces*. The amount of growth produced was somewhat parallel to the amount of glucose decomposed. The actinomyces, however, liberated a greater proportion of the nitrogen in the glycine as ammonia even in the presence of glucose, actually about 50 per cent of the nitrogen of the amino acid decomposed being thus liberated. In the presence of glucose, the fungus apparently utilized most of the nitrogen for cell synthesis.

Various species of actinomycetes have been found (485) capable of attacking rather readily native proteins of both plant and animal origin, such as zein, edestin, gliadin, albumin, and casein. The ratio of the protein decomposed to cell substance synthesized was 9:1 for the actinomycetes, as compared to 22:1 for bacteria and 5.6:1 for fungi.

TABLE 38: *Decomposition of xylan by actinomycetes* (462):—

Organism	Xylan, mg		Mg of ammonia-nitrogen in medium		Xylan decomposed / Nitrogen consumed
	Left	Decomposed	Left	Consumed	
Control	152	–	8.6	–	–
Streptomyces No. 26	39	113	2.3	6.3	18
Streptomyces No. 40	25	127	3.3	5.3	24
Streptomyces No. 50	30	122	2.2	6.4	19

The corresponding ratios of nitrogen in protein to nitrogen in cell material were 20:1, 24:1, and 17.6:1. The actinomycetes were thus found to occupy an intermediate position between the bacteria and the fungi as regards the ratio of protein decomposition and cell synthesis.

Actinomycetes are able to utilize a great variety of carbohydrates, including not only simple sugars and starches, but also hemicelluloses and cellulose. Their action upon the more complex compounds is frequently selective in nature, being limited to certain organisms. In the decomposition of hemicelluloses, for example, actinomycetes were found (462) to be more effective than fungi. This process was influenced by the nature of the carbohydrate and by the environmental conditions. The process of decomposition can be measured conveniently by the amount of CO_2 liberated. In order to decompose the hemicelluloses, the organism requires available nitrogen for cell synthesis, a definite relation existing between the two, as shown in TABLE 38. Some species of actinomycetes are capable of decomposing cellulose very rapidly. Under conditions favoring their development, as in neutral alkaline and arid soils or with insufficient moisture, actinomyces may play an important part in this process.

Krainsky (230) divided all the actinomycetes, on the basis of their ability to attack cellulose, into two groups: *1.* the macroactinomyces, forming large colonies on agar and not decomposing cellulose at all or only to a very limited extent; *2.* microactinomyces, forming minute colonies on agar and decomposing cellulose rapidly with the formation of pigments. Rubentschik (372) demonstrated that certain actinomycetes produce a melanin pigment in their attack upon cellulose. Decomposition of chitin is also carried out by various actinomycetes (398). The formation of a clear zone on an agar plate was designated as chitinolysis. Pathogenic forms are particularly capable of bringing about this process. In addition to the pathogens, saprophytic forms, notably *S. griseus, S. griseolus, S. aureus, S. exfoliatus, S. fradiae,* are able to attack chitins.

In addition to the carbohydrates and organic nitrogenous compounds, actinomycetes are able to decompose various steroids, such as cholesterol (430), a variety of aromatic compounds (156), acetylene (38), and many others. They are able to utilize numerous other organic compounds, such as agar, rubber, paraffins, and lignins, substances known to be fairly resistant to attack by the great majority of bacteria and fungi. Büttner (59), for example, demonstrated that various aerobic actinomycetes are capable of attacking paraffin; decomposition of the paraffin could be measured by the liberation of carbon as CO_2; about one fifth of the carbon was used for cell synthesis. Stanier (408) made a detailed study of the variability in agar-decomposing capacity of *S. coelicolor.* Certain actinomycetes are able to attack building materials and bring about their destruction (277).

Decomposition of Complex Plant Materials:—Actinomycetes can attack various plant and animal residues and bring about their decomposition. In a study of the decomposition of alfalfa by different organisms, Waksman and Hutchings (469) found that 43 per cent of the hemicelluloses and 23.2 per cent of the cellulose were decomposed in 39 days. Nearly 20 per cent of the nitrogen was liberated as ammonia, pointing to considerable protein decomposition, since much of the nitrogen must have been used for the synthesis of cell material. In 50 days only 9.3 per cent of the oat straw was decomposed and this was largely at the expense of the hemicelluloses (24.5 per cent), since only little cellulose and lignin were attacked. Little decomposition of corn stalks took place. When some $CaCO_3$ or phosphate buffers were added, however, the material underwent rapid decomposition. Three pure cultures decomposing an average of 20 per cent of the total dry material, attacked the water-soluble substances most heavily (30.5 per cent), then the hemicelluloses (16.7 per cent) and the cellulose (5.4 per cent). The most striking point, however, was the fact that the decomposition of the lignin in these materials was always associated with the presence of the actinomyces.

On comparing the decomposition of straw by an actinomyces, a fungus, a mixed population, and a complex soil infusion (Table 39), it was found that the actinomyces was capable of decomposing a considerable amount of cellulose and lignin. The cellulose-decomposing capacity of the actinomyces was less than that of the fungus *Trichoderma*. The latter, however, did not attack the lignin, whereas the actinomyces did. The actinomyces synthesized less cell material than the fungus, as shown by the smaller amount of ammonia assimilated.

Table 39: *Decomposition of wheat straw by different microorganisms* (447):—

Organism	CO_2 liberated mg C	Amount of decomposition in milligrams			Ammonia-N assimilated mg
		Pentosan	Cellulose	Lignin	
Streptomyces sp.	–	–	215	32	8.2
Trichoderma sp.	206	201	406	0	27.3
Trichoderma sp.† *Streptomyces* sp.† Bacterium sp.	223	209	434	24	27.1
Soil infusion	376	379	572	2	20.5

† Controls contained 708 mg pentosan, 1,290 mg cellulose, 794 mg lignin and 29.8 mg ammonia-nitrogen.

The decomposition of plant materials by actinomycetes is influenced by a number of factors, such as reaction, aeration, moisture content, and temperature. In a study of the effect of reaction upon the decomposition of dried blood by actinomycetes, as measured by ammonia formation, the maximum activities were found to take place at *p*H 5.8 to 7.7. Some organisms showed some activity at *p*H 5.0, but very little at *p*H 4.0 and *p*H 8.8.

Decomposition of Humus:—In view of their capacity to decompose complex plant materials, actinomycetes would be expected to take an active part in the decomposition of organic matter in the soil. This is true of both nitrogenous and nonnitrogenous materials. Because of

Table 40: *Decomposition of sedge and reed peat by microorganisms* (473):—
On the basis of 20 gm of dry peat decomposed for 28 days

Organism	CO_2 liberated mg C	Ammonia-N liberated mg N	Ratio of C : N
Streptomyces sp.	87.7	11.6	7.6
Trichoderma sp.	88.4	13.3	6.7
Soil infusion	68.7	5.8	11.8

their ability to attack native lignin, actinomycetes might be assumed also to be capable of attacking humus materials. This was actually found to be the case for peat (Table 40). A pure culture of an actinomyces and a strain of *Trichoderma* decomposed more of the peat than the complex soil population, as shown by the greater amount of CO_2 liberated and of ammonia produced. The ratio of the carbon to the nitrogen liberated was less for the pure cultures than for the complex microbiological population, pointing to the fact that the former attacked more of the nitrogenous constituents than did the total population.

Macé has shown that actinomycetes, through their ability to decompose proteins into amino acids and ammonia, bring about the formation of humus in the soil. In view of the fact that actinomycetes synthesize considerably less mycelium than do fungi, only small quantities of nitrogen are assimilated, and most of the nitrogen is liberated free in the

Table 41: *Decomposition of stable manure by pure cultures of thermophilic microorganisms and by a mixed thermophilic population* (459):—

Per cent of dry material left after decomposition

Inoculum	Total dry material	Hemicellulose	Cellulose	Lignin	Protein	Ash
Manure control	100.0	22.8	19.7	18.5	11.0	9.8
Bacteria	80.2	19.4	19.0	23.1	13.3	10.9
Actinomycetes	83.0	18.0	18.5	20.5	11.9	12.2
Fungi	60.7	17.2	12.6	23.6	13.6	14.4
Natural population of manure	37.9	11.7	4.6	18.4	19.9	24.2

form of ammonia. Nonnitrogenous organic materials are utilized for cell synthesis, but they do not exert such a depressing effect upon ammonia liberation by actinomycetes as in the case of bacteria and fungi.

The accumulation of humus in the soil is an index of the great resistance of this group of organic substances to decomposition by microorganisms. Since humus contains the larger part of the soil nitrogen, its decomposition is of great importance to soil fertility. Actinomycetes seem to be capable of attacking this resistant material and bringing about its decomposition. Liming of soil and draining of swampy soil favor the development of actinomycetes and also the decomposition of the soil organic matter. According to Fousek (125), an increase in plant growth is obtained by adding mycelium of actinomycetes to the soil; this is due to increased decomposition of the organic matter thus brought about.

Thermophilic Composts:—As was pointed out above, the actinomycetes comprise a number of thermophilic types. In the decomposition

of plant and animal residues in composts of stable manures or plant materials, the temperature rises rapidly, and the various organic constituents, notably the carbohydrates, decompose rapidly. The numbers of actinomycetes increase rapidly with a rise in the temperature of the compost. It is to be expected, therefore, that these organisms would play an important part in the processes of decomposition taking place in these composts. That this is so, is brought out in Table 41. The degree of decomposition can be measured either by a reduction in dry matter or by the increase in the ash content of the compost, since the ash accumulates with the decomposition of the organic constituents. The greatest decomposition was brought about by the complex natural population of the manure. This was followed by decomposition by pure cultures of fungi. The actinomycetes stood in their abilities to decompose the compost materials midway between the fungus culture and the bacteria.

Of even greater interest is the effect of these organisms on the lignin in the compost. Lignins are highly resistant to microbial decomposition. Together with the proteins they contribute to the formation of black humus in soils and in composts. As has been pointed out, actinomycetes apparently have the capacity to attack these resistant complexes. In the case of the fungi and the bacteria there was an increase in the concentration of the lignin parallel to the increase in ash content and to the decrease in total dry material. The actinomycetes, however, brought about a much smaller increase in lignin content, pointing again to the destruction of this material by the actinomycetes.

Conn emphasized the importance of actinomycetes in the decomposition of organic residues in the soil. His conclusion was based upon observations that the actinomyces colonies developing on an agar or gelatin plate made up 20 per cent of the total number of colonies from cultivated soils, as compared to 37.5 per cent of the colonies from sod soils. The longer the period of time during which grass was grown on a soil, the larger was the percentage of actinomyces colonies. In an experiment on the effect of grass roots on the relative abundance of actinomycetes as compared to the total number of microorganisms developing on the plate, it was found that the actinomyces content of an untreated soil remained almost constant throughout the experiment, namely, about 2,900,000 colonies per gram of soil. In the soil receiving grass roots, the numbers increased to 6,000,000 in 2 weeks and remained nearly at that height for 10 months, or throughout the experiment. The stimulating effect upon the development of actinomycetes was caused both by dead grass roots mixed with the soil as well as by grass growing in the soil.

Chapter X

ACTINOMYCETES AS CAUSATIVE AGENTS OF PLANT DISEASES

Actinomycetes are known to occur in the outer layers of roots and tubers, particularly in the potato plant. According to LUTMAN (268), the filamentous nature of irregular lines and simulating cell walls in potatoes and in other plants is actually due to actinomyces mycelium; the infection is said to occur throughout the leaves, flowers, and other parts of the potato plant. No explanation was suggested for the effect of these actinomycetes on the cell walls of plants. Other observers, however, believed that the impression of filaments was not due to actinomyces mycelium but to the staining of the middle lamella.

For staining purposes, RICHARDS (359) impregnated the potato scab organism with carbol-auramin; when exposed to ultraviolet radiation, the mycelium produced a bright yellow fluorescence. By his method, the sections are stained for 4 minutes in a solution containing 100 mg. carbol-auramin, 3 ml. liquefied phenol, and 97 ml. distilled water. They are washed, destained in a 0.5 per cent solution of sodium chloride in 70 per cent alcohol and 0.5 ml. concentrated hydrochloric acid per 100 ml., washed, and mounted in glycerin. The staining is done at room temperature, and no counterstain is used. The marked contrasts of the stained filaments permit the ready localization and study of the micropathology of the tissue by means of a simple fluorescence microscope. This fluorescence technic appeared to confirm LUTMAN'S conclusion that the filaments are intercellular and grow within the middle lamellae.

Comparatively few plant diseases are known to be caused by actinomycetes. These diseases are frequently known as actinomycoses. The common scab of potatoes and that of sugar beet occupy a prominent place. It has also been claimed (178) that a variety of other plants are subject to infection either by the potato scab organism or by other actinomycetes.

An extensive literature has accumulated dealing with the nature of the causative agent of scab, the course of development of the disease, the effect of environment, especially soil conditions, and methods of control. Only a few selected references to this literature will be cited here.

Potato Scab:—

Method of isolating actinomycetes from scab lesions.—Several pro-

cedures are utilized for the isolation of pathogenic actinomycetes from infected plants. One of these (412) may be described as follows:

Potato tubers are placed for 2 minutes in a mixture of fresh calcium hypochlorite solution in water, made alkaline with sodium hydroxide. The tubers are then removed, and a slice is cut, comprising the lesion together with some of the underlying tissue. This slice is

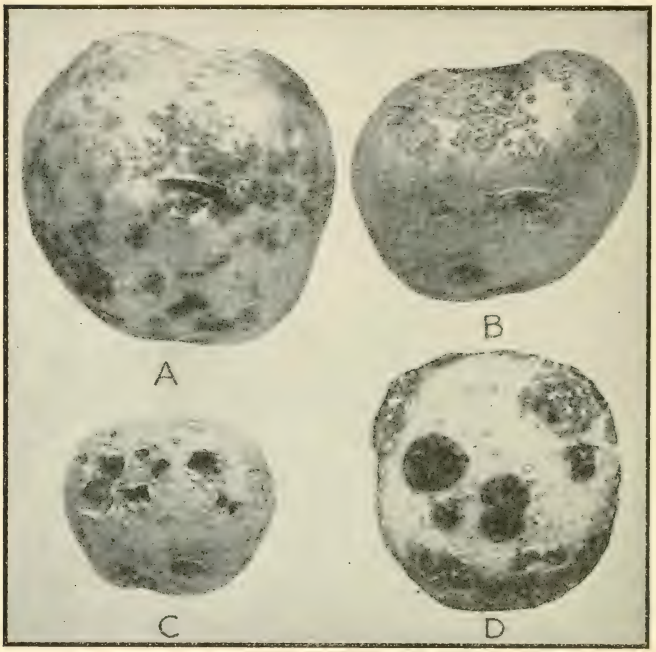

Fig. 32.—Different forms of scab produced by *S. scabies* (*from* Goss, 147).

washed in sterile water and triturated in a flamed mortar with a sterile pestle. The macerated suspension is transferred to a sterile tube and one or two drops placed in the bottom of a sterile Petri dish. About 10 to 15 ml. of egg-albumin agar, which has been melted and cooled to 45°C., is poured into the dish, and the latter is rotated to distribute the suspension through the medium. The plate is incubated at 25-30°C. Colonies of the pathogen develop in 2 days to 3 weeks, depending on the strain of organism.

Causative agent.—The organism causing the common scab of the

Irish potato was at first believed to comprise a single species. It is now generally recognized, however, that a number of species of actinomycetes, all belonging to the genus *Streptomyces*, are capable of causing potato scab. The most important of these species was first described by Thaxter, in 1890, under the name of *Oospora scabies* (415). This name was later changed to *Actinomyces chromogenus*, then to *Actinomyces scabies* (Thaxter) Güssow, and finally to *Streptomyces scabies* (Thaxter) Waksman and Henrici.

It may be of historical interest to cite the original description of the potato scab organism by Thaxter:

"Vegetative hyphae brownish, .06(0.6?)-1.0μ in diameter, curving irregularly, septate or pseudoseptate, branching. Aerial hyphae at first white, then gray, evanescent, breaking up into bacterial-like segments, after having produced single terminal spiral spores by the coiling of their free extremities. Forming a firm lichenoid pellicle on nutrient jelly and usually producing a blackish-brown discoloration of the substratum on which it grows, causing the disease known as scab on potato tubers, and a similar disease of beet roots." This was as perfect a description of a species of *Streptomyces* as could be given at that time.

Since 1890, considerable information has accumulated concerning the nature of the potato scab organism, its morphology, taxonomy, and physiology. A number of problems are still, however, a matter of dispute. This is true of the occurrence of the organism, the presence of several causative agents, and their respective virulence. One of the major reasons for the existing confusion has been the difficulty of producing infection of potatoes or other susceptible plants under controlled sterile conditions.

Scab appears on potato tubers in the form of small brown spots. These increase in size, with several infected spots commonly coalescing, and the underlying tissues becoming brown and pulpy. The infected area is at first smooth and unbroken, but later the skin ruptures, and a shallow depression is exposed. The base and edges of the scab now become thickened with layers of cork laid down by the potato in its attempt to cut off the disease from the underlying tissue.

During the growth of the tuber, the stomata and the state of the lenticels in the apical end offer means of entrance of infectious agent into the tuber. When the lenticels first break open, the parenchyma cells are exposed. This enables the organism to come into direct contact with the cells. Fellows (121) emphasized that growth is essential for infection. Large tubers exposed to the scab organism showed much greater infection than small ones under the same conditions. The pathogen affects the host by extending its influence through the middle lamella of the subepidermal cells. When the host is more mature, it extends through the phellogen. The cells of the latter divide to form new additions to the corky layer. *S. scabies* stimulates cell division. Tubers pass through a period of susceptibility, followed by a

period of immunity. When the growing tubers are no longer forming an epidermis with stomata or young lenticels, the period of susceptibility ceases.

The anatomy of scab formation has been the subject of numerous other investigations (219, 325).

Fully developed scabs vary considerably in appearance, because of different types of infection. In some cases, the depression formed in the early stages of the disease is not raised to the surface by the subsequent formation of cork, thus giving the affected potato a pitted appearance. In other cases, the scabs are raised by the formation of cork, with the result that they stand out above the surface of the tuber in

TABLE 42: *Certain characters of scab-producing actinomycetes* (514):—

ORGANISM	SPOROPHORES	COLOR OF SPORES	COLOR OF STROMA	COLOR OF SUBSTRATE, POTATO	TYPE OF SCAB ON POTATOES (P) OR BEETS (B)
S. aerugineus	Dextrorse	Green	Yellow-brown	Black	Tumulus scab (P)
S. scabies	—	Gray-white	—	—	Deep scab (P)
S. incanescens	Sinistrorse	Light gray	Ochre	Dark violet	Deep scab (P)
S. tricolor	—	Hazel-colored	Yellow-carmine	Blue	Shallow scab (P)
S. xanthostromus	—	Cream-colored	Golden yellow	Brown	Variable scab (P)
S. albus var. *ochroleucus*	—	—	Ochre-yellow	–	Variable scab (P)
S. albus var. *cretaceus*	–	—	Olive-green	Olive	Scab (B)
S. intermedius	–	Light-green gray	Olive-green	Olive	Scab (B)
S. nigrificans	Coremia	Cream-colored	Green-black, ochre		Deep scab (B)

knob-like projections. These two forms of scab are often designated as "pitted" and "raised" (298).

Although numerous bacteria and fungi can be isolated from young and old scabs on potatoes, only the actinomycetes—and these were isolated only from young scabs—were found capable of causing infection (325).

For many years, the idea of THAXTER prevailed that a single organism is concerned not only in potato scab, but in sugar beet scab as well. Detailed studies of the organisms isolated from the various types of scab eventually led, however, to the conclusion that more than one species is involved in the causation of this disease. The multiple origin of scab is now believed to be definitely established. It was even suggested (272) that many actinomycetes found in the soil have patho-

genic tendencies, but, if no host is present, they may lose them. If a suitable host is provided, the pathogenic habits are reacquired, a milder form of scab, such as russetting, appearing first.

In contrast to the concept of multiple species, some investigators believe that not only is the potato scab organism a single species, but that S. *scabies* can cause root necrosis in seedling plants representing many families, notably those of wheat, pea, soybean and radish, as well as a variety of others (178).

Wollenweber was the first to recognize that more than one species of actinomyces is capable of causing potato scab; different types of scab were believed to be caused by different organisms (Table 42). No infection tests on potatoes were made with these cultures. Since most of the species thus described are similar and since large numbers of saprophytic organisms are commonly found in the soil, and are closely associated with scabby surfaces, the pathogenicity of these species is open to question. Wollenweber's descriptions were incomplete and the experimental data were limited.

Millard and Burr (298) isolated 24 cultures of actinomycetes which were said to cause potato scab. Three of these cultures were obtained in duplicate, and only one was found to be identical with Thaxter's original form. The type of scab produced by the various cultures was believed to be correlated with the specific nature of the organisms. The organism identified as S. *scabies* produced the deep scab and was capable of attacking the roots and stolons of the potato plant.

The description of the organisms by Millard and Burr was based on growth in glycerol synthetic solution:

I. Star-like colonies which persist in suspension or cling to the sides of tube:
   1. Deep pigment produced in nearly all media; tyrosinase reaction positive; solid curd formed in brom-cresol milk — S. *clavifer*
   2. Deep pigment produced in protein media only; tyrosinase reaction positive — S. *fimbriatus*
   3. Pale pigment produced in nearly all media; tyrosinase reaction negative — S. *carnosus*
   4. No pigment (or only trace) produced on artificial media; growth on saccharose and dextrose media covered with minute craters — S. *craterifer*

II. Heavy surface growth with abundant aerial mycelium:
   1. Gray aerial mycelium on nutrient potato agar not abundant; fern-like outgrowths produced from margin on saccharose synthetic agar — S. *gracilis*
   2. Aerial mycelium on nutrient potato agar abundant, white; gelatin liquefied, without pigment — S. *praecox*
   3. Liquefaction of gelatin accompanied by production of pigment — S. *setonii*
   4. Carnelian red pigment in calcium malate glycerine agar; abundant aerial mycelium produced on nearly all media — S. *praefecundus*

III. Fair surface growth with some aerial mycelium:
   1. Produces pigment (often green) on all solid media — S. *viridis*
   2. Produces pigment (yellow) on all synthetic media — S. *flavus*

3. Seldom produces pigment and then poor; forms a decided clot in brom-cresol milk *S. wedmorensis*

IV. Scant or no surface growth, but some bottom growth:
1. Produces color changes in brom-cresol milk:
a. Tyrosinase reaction positive; no aerial mycelium on nutrient potato agar *S. scabies*
b. Tyrosinase reaction negative; produces aerial mycelium on most solid media:
a. Abundant aerial mycelium on saccharose synthetic agar
a. Good growth with abundant aerial mycelium on egg albumen agar *S. tenuis*
b. Poor growth with scant aerial mycelium on egg albumen agar *S. marginatus*
b. Scant aerial mycelium on saccharose synthetic agar; no true aerial mycelium on any media, colonies often show dark centers *N. salmonicolor*
2. Produces no color changes in brom-cresol milk:
a. Facultative anaerobe; no true aerial mycelium or only traces on any media; colonies frequently show dark centers *S. maculatus*
b. Obligate aerobe; aerial mycelium arises centripetally on the colonies *S. coroniformis*

V. No growth in glycerine solution; starch not hydrolyzed:
1. Good growth in brom-cresol milk with characteristic color changes *S. spiralis*
2. Poor growth in brom-cresol milk with coagulation, no hydrolysis and no color change *S. sampsonii*

The reports of Wollenweber and Millard that different species of actinomycetes are responsible for the various forms of potato scab were investigated in considerable detail by deBruyn (50), who employed for this purpose the method of Kieszling (218). The sap of different varieties of potato is added to a synthetic medium and inoculated with the various cultures of actinomycetes. Since the *p*H of the sap changes with the ripeness of the tuber, the growth of the organisms in the above medium was parallel to the *p*H change. Best growth was obtained in a sap-medium of a susceptible variety; no growth occurred in a medium to which the sap of young tubers of a resistant variety had been added. The ideas of Wingerberg (511) and Kieszling concerning the existence of physiological scab resistance were thus confirmed. DeBruyn recognized four types of scab, namely, deep, tumulus, common, and superficial or russet scab. Each type of scab was found to be caused by a different actinomyces species, all belonging to the genus *Streptomyces*.

Other investigators, however, notably Schlumberger, Berkner, Goss and Afanasiev, considered the differences in the type of scab to be due to the severity of attack, rather than to differences in the species of the organism concerned. The severity of attack is controlled by external conditions, varietal reactions and virulence of the organism. Attention is to be directed, in this connection, to the fact that even deBruyn recognized that different varieties of potato react differently

to the same strain of the pathogenic organism. The change in type of scab produced by a certain strain of organism was explained by the above investigators as due to a change in virulence or in the conditions which favor growth of the particular organism.

Afanasiev (6) investigated seven parasitic cultures of the scab-producing organism isolated from three different types of scab, namely, the common, deep, and russet forms. Individual potatoes were found to show two or even all three of these types of scab. It was suggested, therefore, that the difference in the scab exhibited by cultures of parasitic actinomycetes was one of degree of pathogenicity rather than of type of organism concerned. All parasitic cultures were able to utilize sucrose and raffinose, whereas most of the saprophytes were unable to use these two sugars. The growth of both parasitic and saprophytic actinomycetes was similar on all media containing different nitrogenous compounds, with the exception of urea. All parasitic and some saprophytic cultures failed to grow on a medium to which 0.5 per cent of urea was added. This was found to be due to the toxicity of the ammonia, which was produced as a result of decomposition of urea. The conclusion was reached that the ability of parasitic cultures to utilize sucrose and raffinose, their inhibition by ammonia, and their ability to produce a melanin pigment in a tyrosine medium could be utilized to differentiate the parasitic actinomycetes from the saprophytic soil actinomycetes.

In a study of the correlation of the cultural characteristics of actinomycetes and scab development, Taylor and Decker (413) isolated 143 cultures, of which 128 were non-acid-fast and 15 were acid-fast or partly acid-fast. The last group did not hydrolyze starch, and 13 of the 15 did not liquefy gelatin, though the others did. Sixty-six of the non-acid-fast produced a dark-brown surface ring of growth on milk; of these, 61 caused typical potato scab. Within the "dark-brown ring" group, variations occurred in the total amount of scab produced, the lesions varying from shallow to deep pits. The work of Schaal (387) on the variation and the physiological specialization of the scab-producing actinomycetes has been discussed previously (p. 76).

Persistence of scab organism in soil.—The parasitic organisms causing potato scab are capable of persisting for a long time in the soil. They thus form a part of the microbiological population of the soil. The parasite has been found (272) even in virgin soils. Pratt (347) obtained a higher percentage of scab on potatoes planted in virgin land in Idaho than on the same land following other crops. He suggested the latter produced some effect upon the soil which resulted in a reduction of the parasitic forms. In a survey of the distribution of the potato scab in Western Nebraska, Goss (146) obtained 67 per cent scab on one virgin soil and 46 per cent average scab on 61 fields never before planted to potatoes when all were planted with the same lot of apparently healthy, treated seed potatoes.

From his use of the contact slide method, Dippenaar (90) concluded that soils rich in organic matter, and containing water equivalent to between one-half and two-thirds of its water-holding capacity, permit the vegetative growth of the scab organism.

In general, the nature and abundance of organic matter, the reaction of the soil, and the presence of antagonistic organisms—all greatly influence development of the scab-producing organism and the amount of scab.

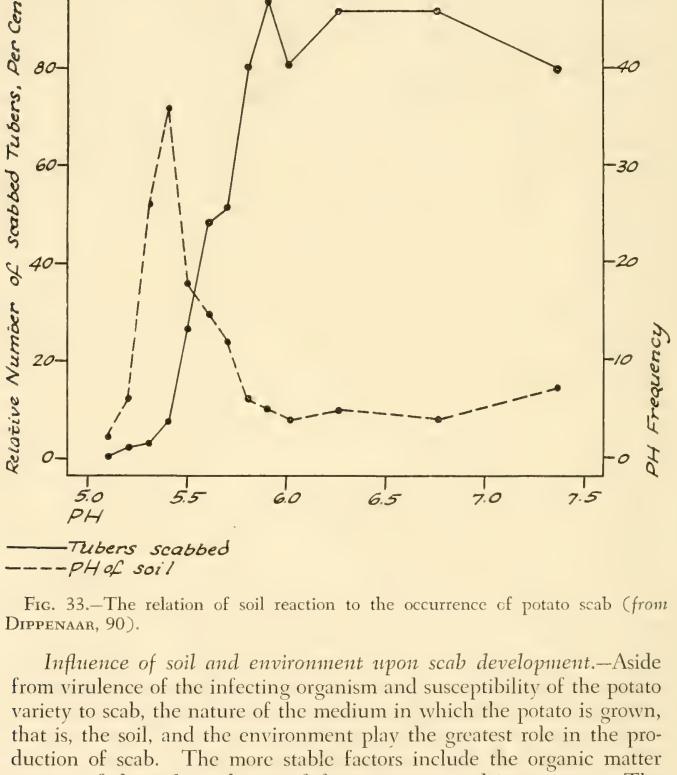

Fig. 33.—The relation of soil reaction to the occurrence of potato scab (*from* Dippenaar, 90).

Influence of soil and environment upon scab development.—Aside from virulence of the infecting organism and susceptibility of the potato variety to scab, the nature of the medium in which the potato is grown, that is, the soil, and the environment play the greatest role in the production of scab. The more stable factors include the organic matter content of the soil, its degree of decomposition and its reaction. The fluctuating environmental conditions comprise three important factors, namely moisture, temperature, and aeration.

Addition of stable manure to soil has been considered to increase the incidence of scab. This effect is believed to be due to the resulting alkaline reaction and accumulation of humus, both of which favor the development of actinomycetes (270). Lime also increases scab production because of the resulting alkalinity. Similar effects are apparently obtained from excessive use of potassium.

Frequently the application of organic materials such as green manures results in the reduction of scab. This may be due to one of the following three factors: (*a*) an increase in soil acidity; (*b*) an increase in the buffering and moisture-holding capacities of the soil; (*c*) a stimulating effect upon microbes which are inhibitory to the growth of the scab organism.

In a detailed study of the effect of soil moisture (287) and reaction (285) upon the development of potato scab, Martin concluded that a high soil moisture and a high acidity are the two important limiting factors. Spore germination is limited by an acid reaction of about *p*H 5.3; it is favored by a higher *p*H value, 8.5 being the maximum. Severe scab can be expected, however, in soils with *p*H values ranging from strongly alkaline to about 5.2. The higher *p*H values of the sap in the tuber and the tendency of the pathogen to change the reaction toward alkaline make growth possible at the lower *p*H values.

Dippenaar believed that the soil reaction is the most important factor involved in a steady increase of the scab organism in soils which are continuously used for potato culture. In soils having a *p*H of 5.2 or lower, the incidence of disease increases very slowly, if at all, with continuous potato culture, provided care is exercised in selection and treatment of the seed tubers.

Soil moisture is also frequently considered as a highly important factor in bringing about an increase or a decrease in abundance of the scab organism in the soil and in the occurrence of scab on potatoes. An increase in moisture increases the yield of the crop and decreases the incidence of disease. This effect may be indirect and may be due largely to the influence upon soil aeration. In general, the effect of soil moisture upon scab development may give quite variable results (116).

Soil temperature is least significant in influencing the occurrence of potato scab. Jones (202) reported that the optimum soil temperature for scab development is about 23°C., whereas the optimum for the percentage of scab to the total tuber surface is about 20.5°C. It was concluded, therefore, that 22°C. is the optimum soil temperature for scab development, under special Wisconsin conditions. Sandford (379) also argued that since the scab organism grows over a wide range of temperature (8° to 38°C.) and since the potato grows and matures between 13° and 32°C., temperature, as it affects host and pathogen, can hardly be considered as a very important factor in the scab problem under average field conditions in most potato growing areas. Most of

the studies on temperature were largely concerned with the effect on growth of the potato prior to infection and were carried out in sterilized soils. In non-sterilized soils and with inoculation at time of tuber formation, there was only little effect of temperature (147).

The actinomycetes that cause scab of potatoes are distinct aerobes. Although the spores are capable of germinating in the presence of only a very low amount of oxygen, the subsequent development of the organisms requires a high oxygen concentration. Spore formation does not take place in the absence of oxygen. The actual amount of oxygen present, rather than the partial oxygen pressure, is the limiting factor both for germination and for growth. An excess film of water will retard germination of the spores on nutrient agar.

Stem necrosis caused by potato scab.—The organism S. *scabies* is also capable of causing stem necrosis. Brown, necrotic lesions are produced on the subterranean stems. These lesions originate at the lenticels or at points of emergence of stolons and secondary roots. In advanced stages, the stem is girdled and rotted at the base with vascular discoloration extending up the stem a distance of 6 to 8 internodes; similar effects are produced on the roots. Varieties of potatoes resistant to tuber scab are also resistant to stem necrosis (179).

Sugar Beet and Mangel Scab:—Besides producing scab on potatoes, certain parasitic actinomycetes are capable of causing scab on root crops, notably sugar beets. Krüger (244) was the first to establish, in 1904, that the production of scab on sugar beets is due to a certain species of actinomycetes. He described several organisms under the names of *Oospora cretacea, O. rosella, O. intermedia, O. tenax, O. nigrificans,* and *O. violacea,* all of which were typical actinomycetes, as we recognize them to-day, belonging to the genus *Streptomyces.* Krüger worked with what he called "girdle" scab of sugar beets; he emphasized that the strains of the parasites which he isolated were not identical with the potato scab organism of Thaxter.

Lutman and Johnson (271) isolated eight strains of actinomycetes from beet scab. Five of these cultures were found to be pathogenic, one being more virulent than the others.

Millard and Beeley (297) recognized two distinct types of scab, the raised and the pitted, on mangels. The raised scab was subdivided into the mound and knob forms, which were found to develop particularly on the yellow-skinned varieties of mangels. A marked difference was found in the origin of the two major types of scab. Pitted scab was similar to the common scab of potatoes. The raised scab was not formed from the cambium of the vascular rings, but resulted from the proliferation of the pericycle. A strain of an actinomyces was isolated from mound scab which reproduced the same type of scab in artificial inoculation experiments. This strain was described under the name of *Actinomyces tumuli.* From pitted scab a strain was isolated which

also reproduced its own type in inoculation experiments. This organism also attacked the roots and rootlets of the inoculated mangel plants, on which it produced numerous characteristic, dark brown, nodular outgrowths. The organism was found to be identical with *S. scabies*. A stock culture also produced the same type of scab in inoculation experiments.

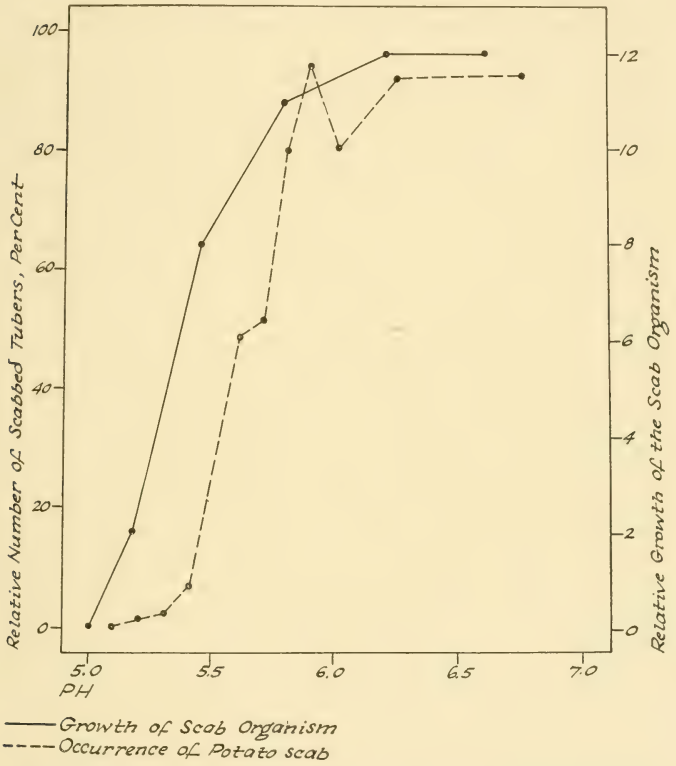

Fig. 34.—Influence of the hydrogen-ion concentration on the growth of the potato scab organism (*from* Dippenaar, 90).

Sweet Potato Pox or Soft Rot.—Soft rot of sweet potatoes was first ascribed to various fungi. Taubenhaus (411) suggested that a certain actinomyces is the causative agent of this disease. The organism, described as *A. poolensis*, was a parasite of superficial wounds following

the pox spots, which were said to be caused by a fungus. Manns and Adams (4, 284) isolated from the sweet potato another organism which they designated as *Actinomyces pox* and which they believed was the cause of the pox. This organism was found to be distinct from *A. polensis.*

A detailed study of the sweet potato disease was made by Person and Martin (289, 336). In heavily infested soils the plants were dwarfed and made very poor growth. The leaves were small and pale green to yellow. Many of the plants died before the end of the season. The root system was poorly developed, most of the roots being entirely decomposed and many breaking apart when the plant was lifted from the soil. Small, elongated, dark-colored lesions also were observed on the stem below the soil line. On mature potatoes the infection produced pits or cavities, with irregular jagged or roughened margins. These lesions varied from one-quarter of an inch to more than an inch in diameter, sometimes coalescing and covering most of the surface of the potato. At first, the lesions were covered by the skin of the root, but when the latter broke away, the slightly sunken pits were exposed. The new epidermal layer was covered with black, granular remnants of the old dead tissue. The severity of rot on sweet potatoes was influenced by the water content of the soil and its reaction. A high water content favored more rapid growth of the roots and enabled a diseased plant with a deficient root system to absorb water and essential mineral salts more easily from the soil. Because of this, rainy seasons enabled infected sweet potato plants to produce vines and often to give satisfactory yields.

The organism causing sweet potato rot was found to be a typical producer of aerial mycelium and was described as *A. ipomoea,* belonging to the genus *Streptomyces.*

Like potato scab, sweet potato rot does not develop in soils of *p*H below 5.2. In soils of *p*H 5.8 to 6.2, however, the disease develops readily. It can be eliminated by adding sufficient sulfur to lower the *p*H to 5.0.

Person and Martin concluded that sweet potato rot is more serious in dry soils and in wet seasons, and it is found in soils with a *p*H above 5.2. The disease has been produced in the greenhouse and in the field in inoculation experiments with pure cultures of *S. ipomoea.* In cultures, the optimum temperature of growth for *S. ipomoea* is 32°C. The optimum reaction for growth is *p*H 5.6 or above.

Other Plants Infected by Scab Organisms.—In addition to potatoes, sugar beets, and sweet potatoes, certain other plants are infected with various forms of scab, which are apparently caused by actinomycetes. These plants comprise turnips, rutabagas, occasionally radishes and carrots, peppers, brazil nuts (406), and a variety of others (165, 178, 332, 360). According to Ken Knight (216), lesions caused by actino-

mycetes are commonly observed not only on the tubers of potatoes (*Solanum tuberosum*), but also on the roots of turnips (*Brassica rapa*) and rutabagas (*B. campestris*); less commonly on the roots of radishes (*Raphanus sativus*); and occasionally on carrots (*Daucus carota*) and parsnips (*Pastinaca sativa*). The eggplant (*Solanum melangena*), the weeds S. *nigrum* and *Amaranthus retroflexus*, and several other plants, such as crucifers, may become scabbed when grown on scab-infested soil. Afanasiev (6) obtained scab formation on radishes and on sugar beets by a strain of S. *scabies*. Species of *Streptomyces* were also found by other investigators in the leaves of strawberry plants, causing such deformities as small-leaf, yellows, cauliflower disease, and chlorosis (23). Infecting agents of the various plants are said to represent different species of actinomycetes, in addition to the S. *scabies* that attacks the Irish potato.

The possibility that an actinomyces is responsibile for gummiosis of citrus associated with wood necrosis has also been suggested (140).

Other Plant Actinomycoses:—In certain plants, actinomycetes produce associations with the root systems which are believed to comprise specific relationships comparable to those of mycorrhiza. Peklo (333) made a detailed study of the endophytes of the alder bush, *Alnus glutinosa*, and of sweet gale, *Myrica gale*. The formation of pseudoparenchymatous tissues in the swelling of the roots of the alder was explained by the condensation of the plasma together with the mycelial mass of the endophyte. Two species of actinomycetes, *Streptomyces alni* and S. *myricae*, were isolated. These organisms produced, in culture, swellings comparable to those formed by animal pathogens. The significance of these associations for plant growth still remains to be established.

Methods of Control:—

Influence of antagonistic organisms.—Millard and Taylor (299) found that certain nonpathogenic organisms in the soil markedly reduce scab. Similar results have been obtained by other investigators (90). The nature of the antagonistic organisms which repress the development of the scab organism appears to vary greatly, and, therefore, more than a single antagonist is involved. Goss (147) also ascribed an important role to competing soil microorganisms in reducing potato scab. Infection of tubers grown in sterilized soil with S. *scabies* could be overcome by inoculation of such soil with an aqueous extract of unsterilized soil.

The soil microflora exerts an important effect in reducing the activity of the infectious agents which result in the production of scab. Sandford (379) believed that the favorable effect of applications of certain forms of organic matter in controlling potato scab was due to stimulation of soil microorganisms which are injurious to the growth

of S. *scabies*. To this action, Millard and Taylor also ascribed the effects of green manure in controlling potato scab. Species of *Trichoderma* were found to have a marked effect upon the growth of the scab organism in artificial culture media; in the soil, however, the toxic substance produced by the fungus may be rapidly destroyed, and the inhibitive action was, therefore, questioned (86).

Certain interesting conclusions bearing upon the above theories can be drawn from the results presented in Table 43. When grown in

Table 43: *Effect of competition of soil microorganisms upon occurrence of scab* (299):—

Treatment of soil*	Number of plants	Number of tubers	Percentage of scabby surface			
			0	0–2	2–25	25–100
Sterilized soil, inoculated with *S. scabies*						
No supplementary treatment	58	249	2	6	34	58
Filtrate of fresh soil	34	176	8	17	27	48
Filtrate of sterilized manure	40	205	7	8	36	49
Sterilized manure	20	102	12	13	49	27
Filtrate of fresh manure	39	215	20	22	34	23
Fresh manure	20	78	54	17	23	6
Penicillium sp.	30	160	1	4	35	60
Bacteria	30	160	9	9	38	44
Streptomyces 91 (saprophytic)	30	103	7	7	48	39
Mixture of microorganisms	19	94	2	2	52	42
Sterilized soil, not inoculated						
No supplementary treatment	20	85	100	0	0	0
Unsterilized soil, inoculated with *S. scabies*						
No supplementary treatment	50	211	17	25	39	19

* All treatments were made at the time of inoculation. The filtrates were obtained by soaking 4 parts of soil or manure in 6 parts of water over night, filtering through cheesecloth, and adding 200 ml per pot. The manure was added at the rate of 200 gm per pot.

sterilized soil, all tubers remained uninfected. When the same soil was infected with S. *scabies*, only 2 per cent of the tubers remained uninfected. The additional introduction of pure cultures of fungi (*Penicillium* sp.), of bacteria, and of actinomycetes (*Streptomyces* sp.) and even of mixtures of microorganisms had comparatively little effect in reducing the scabbiness of potatoes. Filtrates of fresh soil and of sterile manure also had comparatively little effect. However, the addition of fresh stable manure and, to a somewhat lesser degree, of sterilized manure and of filtrate of fresh manure resulted in a considerable reduction in the percentage and in the degree of scabbiness. This may possibly be due to the fact that the manure stimulates the development of microorganisms which are antagonistic to the scab organism.

Cultural methods of control.—Various methods are used for the

control of potato scab. These are based largely upon seed selection, seed treatment, soil fertilization, crop rotation, and soil treatment.

The methods of seed treatment (60, 149, 288) are based upon surface sterilization of the seed by means of various chemical disinfectants. Here belongs the use of hot formaldehyde, of mercuric chloride, and numerous others. Organic mercury compounds have not been found satisfactory (216). In view of the fact that the scab-producing organisms persist largely in the soil, seed treatment has not been universally accepted (35).

The use of acid-forming fertilizers and of sulfur, which result in making the reaction of the soil sufficiently acid to prevent the development of the scab organism, has been found to give the most satisfactory results in the control of potato scab. In using sulfur, it has often been considered advisable to inoculate it with active cultures of the sulfur-oxidizing organism *Thiobacillus thiooxidans* (286). The plowing under of green cover crops combines the favorable effect of reducing scab with that of improving soil. When used in connection with acid-producing fertilizers, the results obtained from such practices are highly satisfactory. The use of stable manures, however, increases the amount of scab.

Rotation of crops is essential when scab infestation is particularly heavy. Several years of alfalfa, for example, preceding the growth of potatoes greatly reduces the amount of scab. Where short rotations are practiced, sweet clover is recommended preceding the potatoes (148).

Since an excess moisture has been found to be injurious to the growth of actinomycetes in soil, one would expect that excessive rainfall would not favor scab development. This is fully justified by the popular expression: "A dry season is a scab year, a wet season is a scab-free year." Heavy watering of crop actually reduces the amount of scab (325). This general impression has frequently been questioned, however, since not only has more severe scab been frequently observed in irrigated than in dry land, but the lower portions of irrigated fields often gave the more severe scab.* The conclusion may, therefore, be reached that the whole complex of soil texture, moisture and aeration is involved rather than merely moisture.

* R. W. Goss. Private communication.

Chapter XI

ACTINOMYCETES AS CAUSATIVE AGENTS OF HUMAN AND ANIMAL DISEASES

Etiology of Infections:—It has now been definitely established that certain diseases are caused by actinomycetes, the anaerobic and aerobic types; the latter comprises both acid-fast and non-acid-fast forms.

The specific nature of the organisms that are capable of causing actinomycotic infections in animals and man has aroused considerable discussion and has often been the cause of much confusion. Despite the fact that an animal pathogen, *A. bovis,* was among the first actinomycetes ever described and has given the very name, *Actinomycetales,* to the order of the organisms, and despite the very extensive literature that has accumulated on the pathogenic nature of certain actinomycetes, the identities of the specific agents that cause actinomycotic diseases in man and in animals have been the subject of much disagreement. No attempt will be made here to review this literature, an early summary of which was made by Musgrave *et al.* (310). Attention will merely be directed to some of the more recent investigations.

Wright (519) made a detailed study of the cultural characteristics of a number of isolates of disease-producing strains of actinomycetes from animals and from man. He came to the conclusion that only a single anaerobic species was involved. He believed that the reported isolations by Bostroem and others of aerobic forms responsible for disease conditions were merely contaminations. These conclusions were drawn by many other investigators. The very extensive literature, however, on *Nocardia asteroides* definitely pointed to the fact that actinomycetes causing infections in man and in animals comprise both anaerobic and aerobic organisms. The existence of various types of clinical actinomycosis has been emphasized. This is brought out, for example in the work of Naeslund, as summarized in Table 44.

Henrici (169) recognized three well-defined types of infection in man and in animals caused by actinomycetes: (*a*) the lumpy jaw type, which is the most common infection and is produced by an organism belonging to *A. bovis,* first isolated by Israel; (*b*) the madura foot type, caused by an aerobic form which is usually designated as *Nocardia madurae* (438); (*c*) the rare infection type, comprising *N. asteroides* (109), which is found most frequently in man, and *N. farcinica* (324), which occurs in cattle.

Schabad (388) considered the existence of another group of microorganisms which stand midway between the actinomycetes and the tuberculosis group and which are capable of causing infection in man. These were looked upon as atypical actinomycetes, causing diseases similar to actinomycosis. The organisms are acid-fast and produce no swellings.

As more accurate information accumulated concerning the specific nature of those actinomycetes that are causative agents of disease, and

Table 44: *Comparative morphological and physiological properties of two common types of pathogenic actinomycetes* (80, 312):—

	Actinomyces A	*Actinomyces B*
Hyphae	Rather long in the body, shorter on artificial media	Long both in body and on artificial media
Aerial spores	Never observed	Very common
Acid-fastness	Seldom	Often
Granules in body	Characteristic	Common
Clubs in body	Characteristic	Less common
Growth on common media	None or only insignificant	Very good
Oxygen requirement	Preferably anaerobic	Preferably aerobic
Growth at room temperature	Absent or limited	Excellent
Pigment formation	Not marked	Reddish or yellow colonies
Resistance to drying	Weak	Very good
Pathogenicity to animals	Subcutaneous injection in cattle sometimes causes limited suppurative swelling. Pathogenicity for dogs and guinea-pigs insignificant	Subcutaneous injection in dogs and guinea-pigs usually produces a local well limited suppurative swelling
Development outside of infectious area	Mouth and other body cavities, such as intestinal canal, not in free nature	In free nature

as a better knowledge was gained concerning the systematic position of this group of organisms, a clearer understanding of their relation to the various types of infection gradually evolved. Just as Wright and others tried to study the relationship of actinomycetes to disease by considering all the organisms involved as comprising only one type or species, so have others assumed the existence of numerous types of infection.

Erikson and others, for example, believed that even the anaerobic organisms should be divided into two groups, the human and the bovine types. Lentze (257) also came to the conclusion that in human and animal actinomycosis two different, largely anaerobic, gram-positive organisms were concerned: one growing on the surface of the medium in

Fig. 35.—Vegetative mycelium of a pathogenic anaerobic actinomyces (*from* Naeslund, 313).

the form of leathery irregular colonies and producing a sediment in liquid media; the other producing smooth, easily broken colonies on solid media and causing turbidity in anaerobic liquid media. These two types were designated as the R- and S forms, the first representing the classical type of Wolf-Israel, and the second resembling typical corynebacteria. Wright, however, believed that no significant differences existed between human and bovine strains (*see* p. 176).

The aerobic disease-producing actinomycetes were believed to comprise an even larger number of organisms. The confusion thus created was largely due to the difficulty of distinguishing between pathogenic and saprophytic types. It is important to remember that pathogenic forms are found only rarely among naturally occurring actinomycetes. Serum reactions for establishing different types among disease-producing actinomycetes have not always given the most satisfactory result.

True actinomycosis is caused by an anaerobic or a microaerophilic species. This organism brings about the formation of granulation tissue and pus which contain the characteristic "Drusen" or "sulfur granules." These granules consist of masses of hyphae which are arranged radially and terminate in the form of eosin-staining clubs, consisting of material which ensheathes the hyphal tips. Certain actinomycetes are keratophytes in nature and have the ability to infect the human skin (317).

Earlier Investigations:—Bollinger (42) was the first to observe, in 1877, the occurrence of an actinomyces in the pus from the swollen jaw of a cow affected by "lumpy jaw." The botanist Harz examined the filaments and the "sulfur granules" and proposed the name *Actinomyces bovis* for the organism with its ray-like growth. Harz did not, however, obtain pure cultures of the infective agent. Ponfick (344) recognized actinomycosis in man in 1882, although the first detailed clinical account of the infection in man was reported by Israel (181) in 1885, on the basis of 38 cases.

Wolf and Israel (512, 513) were the first to make a careful and comprehensive study of the organism concerned in actinomycosis. They isolated from maxillary actinomycosis in cattle a culture which they found to be a microaerophilic form, identical with the *A. bovis* of Harz which is capable of growing at room temperature but grows better at 30° to 37°C. Numerous minute, isolated dewdrop-like colonies appeared on the surface of anaerobic agar slant cultures, the largest colony being the size of a pinhead. The colonies gradually became larger and formed ball-like, irregularly rounded, elevated nodules. The colonies did not become confluent, and an apparently homogeneous layer of growth was seen to be made up of separate nodules. In some cases, the colonies presented a prominent center with a lobulated margin, appearing in the form of rosettes. In stab cultures, growth was sometimes limited to the lower portion of the line of inoculation or was more pronounced there. In liquid broth, growth appeared under aero-

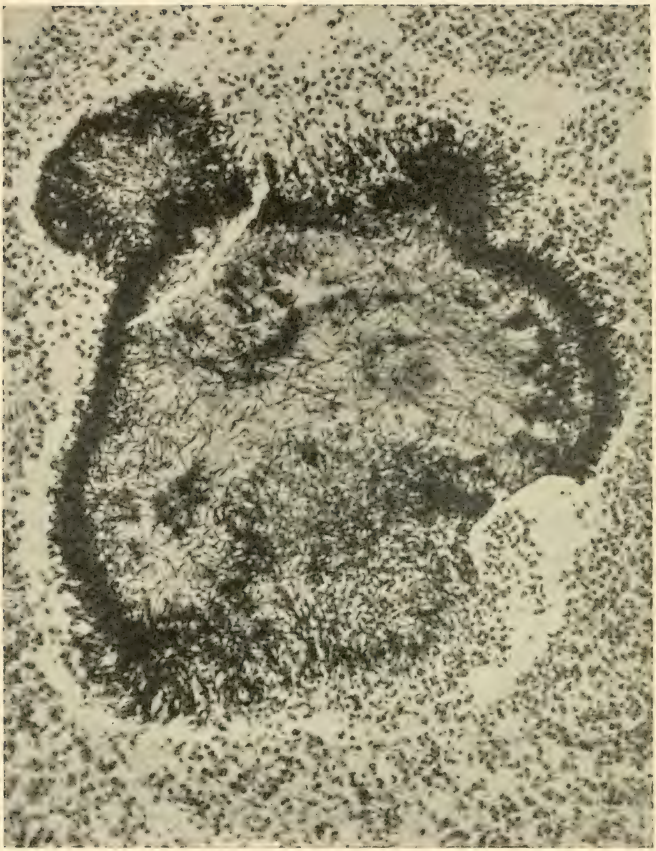

Fig. 36.—Actinomycosis of lymphatic gland showing granule with mycelial network and peripheral growing edge (*from* Cope, 80).

bic conditions in the form of small white flakes, some floating and some collected at the bottom of the tube. Growth was better under anaerobic conditions.

In smear preparations from agar cultures, the organism appeared chiefly as short, homogeneous, usually straight, but also comma-like or bowed, rods of varied length and width. The ends of the rods often showed club-like swellings. Microscopically the growth was characterized by development of long filaments forming a network. The longer filaments were arranged more or less radially; they were straight, wavy, or spiral, and sometimes branched.

On autopsy, infected animals showed tumor growths mostly in the peritoneal cavity and in one instance in the spleen. The tumors were situated partly on the abdominal wall and partly on the intestines, in the liver, and in other tissues. Microscopic examination of the tumors showed in all cases but one the presence of typical actinomyces colonies, in most cases with typical "clubs." The general histological appearance of the tumors was like that of actinomycotic tissue.

Simultaneously with the work of Israel and Wolf there appeared, in 1890, a contribution by Bostroem (44) reporting his isolation of an aerobic organism, which he considered to be the same as *A. bovis* Harz but which was later believed to be a saprophyte. Bostroem's theories were supported by Pinoy (339) and by others who suggested the occurrence of two forms of actinomycosis in cattle, one caused by anaerobic forms, and the other by an aerobic species. Careless isolation techniques and erroneous identification of isolates added to the confusion. Aerobic strains of several types mislabeled *A. bovis* gradually found their way into the culture collections of the world, and came to be known as *A. bovis* (349). Since these strains grow readily, they survived where strains of the anaerobe died and became permanent sources of confusion.

Berestneff (32) differentiated actinomycosis from pseudo-actinomycosis. The first was considered to be typical, in the sense described by Bollinger and Harz and by Israel and Wolf. The second was looked upon as an atypical form and was believed to be caused by various gram-positive and gram-negative bacteria and by those actinomycetes to which the aerobic form of Eppinger and others belongs. Berestneff looked upon *A. bovis* and *A. hominis* as collective names, and believed that actinomycosis is caused by various actinomycetes. Only in very few cases was a single actinomyces found to be responsible for clinical actinomycosis; in most others, a complex population, the members of which belong to the anaerobic flora of the mouth (137), was said to be responsible for the infection.

Nocard first described, in 1888, a pathogenic actinomycete of the aerobic type. This organism was found to be the case of "farcin du boeuf," a disease of cattle in Guadeloupe Islands. De Toni and Trevisan, in 1889, designated the aerobic organism *Nocardia*, in honor of its discoverer, the species being *N. farcinica* (324). In 1890, Eppinger

described a filamentous organism found in the pus of a cerebral abscess as *Cladothrix asteroides;* this organism was transferred to the genus *Nocardia* by Blanchard in 1896 (39). In 1902, MacCallum (274) reported that *N. asteroides* produces a diffuse peritonitis in experimental animals. The characteristic ray fungus granules, consisting of elongated cylindrical structures with laterally radiating clubs, were found throughout the animal body. Generally, however, granules are not considered to be characteristic of *N. asteroides* infection.

It may be of interest to note here that Benbow, Smith and Grimson (30) reported that about 90 per cent of all clinical cases of actinomycosis are caused by *A. bovis.* The remaining 10 per cent are caused by *Nocardia,* some of which are acid-fast or partially acid-fast.

Wright, in 1904, made a detailed study of actinomycosis in man and in animals and of the causative agents involved. He suggested that the designation "actinomycosis" be restricted to a suppurative process combined with granulation tissue formation, the pus of which contains the characteristic granules. These are composed of dense aggregates of branched filamentous microorganisms and of their transformation or degeneration products. The latter include the characteristic club-shaped bodies radially disposed at the periphery of the granule. Wright emphasized that cultures isolated from human and bovine cases show no difference which would be sufficient to justify their classification as separate species. He did not accept the prevalent belief, based on the work of Bostroem, Gasperini, and others, that the specific infecting agent of actinomycosis is to be found among certain branching microorganisms, which differ profoundly from *A. bovis* in having spore-like reproductive elements. He suggested that these be grouped together as a separate genus under the name *Nocardia,* and that those cases of undoubted infection caused by them should be designated as "nocardioses" and not as "actinomycoses."

Wright believed that *A. bovis* is a normal inhabitant of the secretions of the mouth cavity and of the gastrointestinal tract, both of man and of animals. In these secretions, it exists not in the characteristic forms seen in the lesions, but as fragmented filaments growing in company with bacteria. He also suggested that foreign bodies so frequently found in actinomycotic lesions are not carriers of the organism into the tissues from without, but that their traumatic and irritative effects furnish a point in the tissues for the *Actinomyces* to enter with the secretions from the mouth cavity and from the gastrointestinal tract. Here the organism develops into characteristic colonies and produces actinomycotic lesions.

Recent Studies:—A very extensive literature has now accumulated on the etiology of infections caused by actinomycetes. These are usually grouped under the anaerobic and aerobic types, as brought out

in the work of Naeslund. The first or the A form was readily isolated from the mouth. It proved to be the typical *A. bovis* and could bring about the true actinomycotic infection. The B form was a pathogenic aerobe, considered to be less important than the anaerobe. It was commonly found in nature, usually producing reddish or yellowish colonies, was acid-fast, and usually formed spores. The properties of these two types, as summarized by Cope, are given in Table 44. Cope (80) reviewed 1330 cases of actinomycosis. Of these, 56.8 per cent affected the cervicofacial region, 22.3 per cent the abdomen, 14.9 per cent the thorax and 5.9 per cent other sites.

Lord (265) demonstrated the presence of actinomycetes in sputum and in the contents of 16 carious teeth. Emmons also cultivated organisms of the *A. bovis* type from the oral cavity. Slack (403) presented a detailed discussion of the exogenous *vs.* endogenous theory of infection in actinomycosis: the first is supported by the fact that awns of grass and grain are frequently observed in actinomycotic lesions; the second is supported by the fact that anaerobic cultures of actinomycetes have been isolated from normal mouth, from tonsils, from carious teeth and from pyorrhea pus. The oral cavity was looked upon as the source of infection, possibly accompanied by sensitization.

Rosebury (368) believed that one and the same organism is the etiologic agent of maxillary actinomycosis in man and of the appendix, pleura, and reproductive organs. Emmons (108) isolated from the tonsils, in pure cultures, two microaerophilic types of actinomyces: one, morphologically and physiologically similar to *A. bovis;* and the other somewhat different morphologically, but also considered as a strain of *A. bovis.* Magnusson (283) and Negroni and Bonfiglioli (318) also reported considerable variation in the strains isolated from clinical actinomycosis.

True Actinomycosis:—Actinomycosis has often been confused with other infections, as shown by the variety of names applied to it, such as "streptothrichosis," "sporotrichosis," "nocardiosis." This disease affects both man and cattle, usually involving the jaw. Because of this, "lumpy jaw," "pig jaw," and "wooden tongue" are terms frequently applied to the disease. It is not contagious, but once acquired is difficult to eradicate. It is characterized by a swollen jaw and a hard board-like induration, accompanied by destruction of the normal tissue and the formation of granulation tissue. The "sulfur granules" present in the pus consist of cellular debris and radially arranged hyphae. The hyphae terminate at the periphery in "clubs" which are composed of eosinophilic material forming a sheath around the hyphal tip. This phenomenon has been looked upon by some as characteristic of the particular disease condition. Emmons, however, emphasized that whereas other infections also give rise to clubs, certain forms of actino-

mycosis such as those caused by *N. asteroides* and *N. madurae* do not produce clubs; even *A. bovis* may not form clubs in some tissues, under certain conditions.

Emmons (106) defined actinomycosis as "an infection caused by invasion of the host by some species of *Actinomyces*." Several forms were recognized, namely, actinomycosis of the skin, actinomycotic

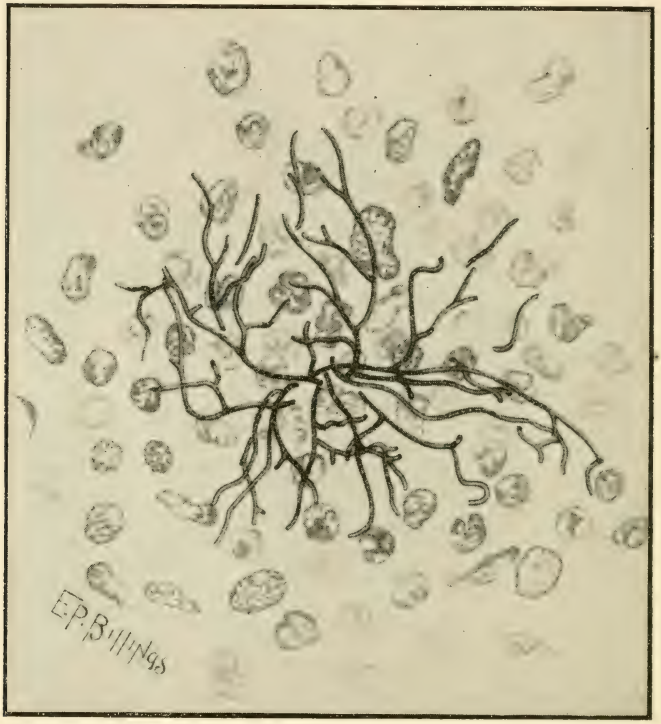

Fig. 37.—An early study of the structure of an actinomyces colony from a bronchopneumonic nodule (*from* Butterfield, 58).

meningitis, lung infection, Madura foot, actinomycotic types of mycetomas. The presence of sulfur granules is frequently considered as a diagnostic symptom of actinomycosis. Granules may be produced, however, by other organisms, and some actinomycetes do not form granules on clubs (496). Club formation is frequently considered (24, 276, 519) as evidence of diminished growth of the host, the presence of

clubs in the tissues depending upon a balance between the host and the parasite.

The mouth is commonly looked upon as the source of infection of maxillary actinomycosis (519). The contents of the mouth and tonsils were reported (266) capable of causing actinomycosis in experimental animals; this led to the conclusion that *A. bovis* is often present in oral cavities, where it may exist as a saprophyte. In spite of his emphasis upon the specificity of *A. bovis* as the major agent of actinomycosis, Emmons suggested the possibility that there are also atypical strains which are found in certain lesions, in sputum, in carious teeth, and in tonsils.

In 47 per cent of tonsils examined, the presence of filaments of an actinomyces was demonstrated (107). Microaerophile cultures were obtained in 23 per cent of the cases, the organism corresponding to *A. bovis*. The conclusion was reached that this organism exists as a saprophyte in the granules sometimes formed in the crypts of the tonsils. Upon discharge into the oral and digestive cavities it may invade more susceptible tissues and give rise to true actinomycosis. In addition to the mouth organs (jaw, tongue) actinomycosis may also affect the abdominal organs, usually the appendix, the lungs, and in the cow, the udder (110).

Anaerobic strains of actinomyces were isolated by Lord and Trevett (267) from the contents of carious teeth. After isolation, however these strains grew aerobically. The work of Naeslund on the presence of the anaerobic form in the normal mouth has already been mentioned. To this must also be added the observation of Magnus concerning the occurrence of anaerobic actinomycetes in the pharynx and nasopharynx.

Rosebury also considered the mouth and throat as the natural habitat of the pathogenic *A. bovis*. This organism was looked upon as comprising an obligate parasite. Sharp differentiation was made between the parasitic "lumpy jaw" and the saprophytic actinomycetes, as brought out in Table 3. The "mycetomas" were included among the saprophytic types.

Rosebury isolated 15 strains of *A. bovis;* 4 of these were obtained from cervicofacial actinomycosis and 11 from gingival scrapings taken under oral pathological conditions in the absence of actinomycosis. Streaked plates of brain heart infusion agar were used for isolation purposes. Optimum conditions for continued growth of these organisms were provided by anaerobiosis in the presence of 5 per cent carbon dioxide. Some strains were capable of making limited growth, however, in air. Considerable variation was observed in oxygen tolerance among the different strains, at different times. Pure cultures were maintained by cultivation under anaerobic conditions with carbon dioxide and by alternate transfer through different culture media.

Of 45 animals inoculated by different routes, experimental acti-

nomycosis was obtained in only 5, namely, progressive and fatal actinomycosis in 2 guinea pigs and in 1 rabbit, and localized and benign forms in 2 other rabbits. Other repeated intravenous or intraperitoneal injections of large doses of organisms seemed to be innocuous. Single or repeated subcutaneous injection usually produced only mild local lesions, from which the organism could seldom be reisolated. By intrapleural injection and by inclusion, in the inoculum, of sterile pulverized salivary calculus, fatal reactions were obtained irregularly.

The conclusion was reached that results thus obtained are in accord with the view that actinomycosis is an endogenous infection. The negative results frequently obtained in animal experiments appeared to emphasize the function of unknown factors in the pathogenicity of actinomycosis. Malignant actinomycosis could not be explained either by repeated inoculation or sensitization. The concept of allergy to the organism as a cause of progressive actinomycosis was eliminated. The capacity of *A. bovis* to survive and multiply in the tissues was shown to be of a very low order. The organism did not establish infection when introduced in repeated massive doses by the intravenous or intraperitoneal routes; local lesions produced by subcutaneous inoculation underwent rapid autosterilization. The occasional ability of the organism to persist and proliferate in the tissues was believed to be conditioned by as yet undisclosed factors of altered host resistance.

The positive results obtained by Rosebury on inoculation of *A. bovis* mixed with sterile pulverized salivary calculus were believed to embody direct evidence that this calcified deposit on the teeth may play a part in the pathogenisis of actinomycosis. According to various investigators, including Naeslund, these masses contain *A. bovis*, which seems to form the stroma upon which the masses are deposited.

Actinomycetes of the *A. israeli* type can be readily demonstrated in broncho-pulmonary infections. In a study of 240 patients reported by Kay (209), 109 cultures were isolated from the sputum, 65 from bronchoscopic specimens, and 6 from long abscess exudates. The organism was believed to have less influence on the clinical course and prognosis than the mechanical factors.

Downing and Conant (94) also came to the conclusion that the source of infection with *A. bovis* is endogenous. The fact that this organism was isolated from tonsillar crypts and carious teeth have established the human mouth as the habitat of this anaerobic species. They recognized 4 types of actinomycosis:

1. The cervicofacial type caused by *A. bovis*, probably the most frequent clinical form of the disease. Tooth extraction or infection referable to the teeth is followed by an infection through the tissues of the lower jaw and neck. Extensive cellular infiltration of the lesion produces a tumor-like hard mass, from which abscesses rupture, leaving multiple draining sinuses. Occasional extension of the infection to the cranial cavity results in brain abscesses.

2. Pulmonary actinomycosis is characterized by the chronic nature of the

infection. Metastatic cerebral abscesses or meningitis is a frequent complication of primary lung infection.

3. Actinomycosis of the central nervous system usually does not present symptoms referable to a distinct clinical entity.

4. Abdominal actinomycosis. This form usually originates in the region of the appendix and cecum, presenting the picture of subacute appendicitis. The disease may follow appendectomy, with failure of the scar to heal completely or a breaking through of a sinus in the old scar or in the region of the navel.

The causation of infection and the pathology of actinomycosis are discussed in further detail by Cope (80) and by Poncet and Berard (343), Henrici (169), and many others. The exogenous theory of

Fig. 38.—The appearance of a *Nocardia* in the sputum of an infected patient (*from* Davis, 88).

infection at first based upon the work of Bostroem, who suggested that infection results from handling of straw or other plant materials, has been gradually replaced by the endogenous theory, as postulated by Lord, Naeslund, Emmons, Slack, and others. According to the

newer theory, the mouth, including the oral cavities and pyorrhea pus, the pharynx, and other organs such as the tonsils, is the normal carrier of the infectious agent.

In addition to the common abdominal, pulmonary, and cervicofacial, forms of actinomycosis, other types exist, such as cardiac involvements and subcutaneous infections. Myocardial and pericardial forms of actinomycosis may be considered clinically as cases of rheumatic heart disease (81). Two cases of actinomycotic endocarditis, due to aerobic forms, have been studied in detail by Wedding (496). Gins and Paasch (137) found that most of the clinical cases suspected of being actinomycotic turned out to be due to other causes; only 1 out of 14 cases was caused by a true actinomyces.

Aerobic Actinomyces Infections:—In addition to the anaerobic forms of actinomycosis, many infections in man and in animals are caused by aerobic species of actinomycetes. "Streptothricosis" or "nocardiosis" should always be differentiated from "actinomycosis," especially in lung infections.

Bostroem (44) reported the discovery of an aerobic type of *Actinomyces*, designated as *A. hominis*, but, as pointed out above, his conclusions were erroneous. The significance of these results has frequently been questioned (170) for several reasons: (*a*) saprophytic aerobic actinomycetes occur abundantly as air contaminants; (*b*) Bostroem succeeded in making only relatively few isolations; (*c*) he, as well as others, failed to obtain infection in experimental animals and in cattle; and (*d*) he made his isolations from the common "lumpy jaw" type of bovine actinomycosis known to be caused by an anaerobe, *A. bovis*.

It may be mentioned here, in passing, that Bostroem's *A. bovis* and several of the forms accepted by Baldacci are probably species of *Streptomyces*.

The aerobic nature of the actinomycetes causing certain infections, such as that of Madura foot spoken of as *Mycetoma pedis* and occurring largely in the tropics, is well established. The pus contains white or yellowish granules, similar to the sulfur granules of the lumpy jaw, from which as many as 13 strains of *Nocardia* have been isolated. The causative agent studied by Vincent (438) is considered to be the most common. The organism is readily cultivated and is now recognized as *Nocardia madurae*. These aerobic organisms cause specific types of mycetomas; their multiplicity has no bearing whatsoever on the early erroneous work of Bostroem on the etiology of "lumpy jaw," as brought out above.

Among the aerobic forms, the acid-fast actinomycetes are particularly significant. Infections of the lungs and of the skin are frequently produced but no clubs are formed at the extremity of the hyphae in infected tissues. The aerobic types are cultivated much more readily than the anaerobes and are pathogenic to laboratory animals. The or-

ganism can be demonstrated in the sputum of infected animals. *N. farcinica,* isolated from cattle, forms a yellowish, wrinkled growth on solid media. *N. caprae,* isolated from the lung of a goat (402), gives a more whitish growth and greater fragmentation of the mycelium. *N. canis* produces infection in dogs (310), and is similar to *N. caprae.*

Eppinger, in 1891, reported the isolation of an aerobic, gram-positive, acid-fast actinomyces from the cerebral abscesses and meningeal exudate of a man who became delirious and died in 2 weeks. This organism readily grew on ordinary media in the form of small colonies that were star-like because of the radiating filaments. It has been known since its isolation as *Cladothrix asteroides, Streptothrix eppingeri, Streptothrix asteroides, Oospora asteroides, Actinomyces asteroides,* and *Nocardia asteroides.* This group of the aerobic pathogenic actinomycetes is the most common. It is the least proteolytic and produces a yellowish to orange, wrinkled growth on solid media; aerial mycelium is white and scant, if formed at all.

In 1921, Henrici and Gardner collected 26 cases of infections with aerobic acid-fast actinomycetes. They reported that the causative organisms fell into three different types, which differed chiefly in the color of the growth on solid medium and in other minor biologic characters. Twenty-three of these cases were of pulmonary origin, and all but one were fatal. Another form was isolated from the sputum of a patient with a cough of 3 years' duration. It differed somewhat from the other three types, and because of the chalky white appearance of the growth, was named *Nocardia gypsoides.* After repeated subcultures, however, the strain became almost identical with *N. asteroides.*

Gordon and Hagan (144) found that some acid-fast actinomycetes isolated from soils and plant material are similar to those found in lesions of men and animals. The pigments produced by these organisms range from yellow through orange to coral. One of the soil forms was pathogenic to rabbits soon after isolation, but not to guinea pigs (145).

Various strains of *Nocardia* have been described as causative agents of madura foot, a disease usually referred to as "nocardiosis" or "madura-mycosis." These organisms include not only *N. madurae,* but also *N. indica, N. pelletieri, N. mexicanus, N. brasiliensis, N. paraguayensis,* etc. These strains were identified by their cultural characters on different media, production of pigment, by their morphology and staining properties, some being acid-fast. They were all apparently of the same general type as the other forms of *Nocardia* listed above.

The same is probably true of the several strains isolated by Pipper and Pullinger (338), namely *N. transvalensis, N. africana* and *N. pretoriana.* These authors assumed that the natural habitat of *Nocardia* is grass. They ascribed, therefore, the relative frequency of *Nocardia* infections in South Africa to local conditions and habits, namely abundance of grass, open-air life, scanty clothing and bare foot

walking. The organisms were said to produce typical clubs, but their cultural characters and animal pathogenicity were different from those caused by species of *Actinomyces*. The nocardias were found to have a marked affinity for iron.

Baldacci (18-20) suggested that the various strains of aerobic acid-fast actinomycetes represent only minor differences in their biologic characters, and must be considered as variants of *N. asteroides*.

According to Drake and Henrici (96), *Nocardia asteroides* has little invasive power for rabbits and guinea pigs. Large doses intraperitoneally in guinea pigs, smaller doses intravenously in rabbits, produced death with acute peritonitis and multiple miliary abscesses, respectively. But smaller doses produced neither lesions nor death. Subcutaneous and intramuscular injections did not spread, but healed. All attempts to produce a progressive disease similar to tuberculosis failed. An allergic state in rabbits and guinea pigs against *N. asteroides* could be induced with regularity only by the intratesticular injection of oil suspensions of live organisms.

Various other strains of aerobic actinomycetes have been isolated from human infections. An actinomyces isolated from the ear involved a disease resembling the ordinary type of chronic suppurative otitis media (327). The organism was said to have maintained itself in the middle ear for 10 or more years. Two cases of *N. asteroides* infection with pulmonary and multiple subcutaneous abscesses and sinuses, with a cerebral abscess suspected in one case, were observed (30). The organisms were isolated from the sputum and from the subcutaneous lesions of both. *N. asteroides* was also isolated (37) from a case of chronic suppurative pneumonitis and massive cerebral abscess in a man under observation for a brain tumor.

Kirby and McNaught (220) studied two cases which showed gross and histologic signs of specific lesions as of an acute pyogenic inflammation with a central zone of liquefactive necrosis and numerous polymorphonuclear leucocytes. About the area of liquefaction there was a zone of granulation tissue with neutrophils, lymphocytes, and plasma cells and, at times, varying amounts of slightly more dense fibrous tissue. The dispersed mycelia of the organism were not seen in hematoxylin and eosin stains but were observed in sections stained by Gram's method.

In one case, the etiologic agent was isolated from the sputum, subcutaneous abscesses, and blood stream; at autopsy, the lungs, peribronchial lymph nodes, heart, thyroid, kidneys, spleen, intestines, muscles, and subcutaneous tissues contained abscesses produced by *N. asteroides*. The second case was hospitalized for 5 days prior to death with a clinical diagnosis of an intracranial lesion without localizing signs. Autopsy revealed an abscess in the cerebellum caused by *N. asteroides*. The lungs were suspected as the primary focus, and no other metastases were found.

N. asteroides, or closely related strains have been isolated from cases of diffuse peritonitis, of pseudotuberculosis with cerebrospinal meningitis, and of brain abscesses. Sartory and Bailly (382) isolated a culture from the urine of a patient suspected of renal tuberculosis. The organism was acid-alcohol-resistant; was cultivated on ordinary solid or liquid media only with difficulty; and grew well on serum and blood media at 35°-37°C. The organism, described as *A. serophilus,* was believed to be the causative agent of renal actinomycosis.

Various attempts have been made to study the immunological reactions of actinomycetes. Goyal (151) examined 11 cultures obtained from collections and as fresh isolations. Most of them appeared to be members of the genus *Nocardia.* When inoculated into rabbits, they proved to be either entirely non-pathogenic or only slightly virulent, except *N. eppingeri.* These cultures were grown in glycerol broth at 38°C. for 30 days. Extracts were prepared in a manner comparable to tuberculin. These extracts were designated as *streptothricine.* Their antigenic reactions were very similar to tuberculin. Animals sensitized to the nocardia extracts were also sensitive to tuberculin, and *vice versa.* Serologic studies confirmed the conclusions reached on the basis of allergy tests; a common antigen was demonstrated for the tubercle bacillus, the diphtheria organism and the nocardias. These results led to the conclusion that there is a definite antigenic relationship between the actinomycetes and the mycobacteria.

Chemotherapy of Actinomycosis:—In addition to the application of vaccinotherapy, radiotherapy, and chirurgy of actinomycosis, subjects which need not be discussed here, extensive use is made of chemotherapy.

A detailed survey of the various clinical aspects of actinomycosis in man and of methods of treatment was made by Colebrook (73), Cope (80), and others. Lyons, Owen and Ayers (273) and others (128) reported favorable results from the treatment of actinomycotic cases with sulfonamides, especially sulfadiazine, or with thymol (248). Long-continued drug therapy is required, and the danger of recurrence is always present. The favorable effect of massive doses of penicillin has also been observed in a number of cases.

Cutting and Gebhardt (82) found sulfadiazine and sulfathiozole more effective than sulfonamide in inhibiting the growth of both anaerobic strains of both laboratory and freshly isolated strains of an organism designated as *A. hominis.*

Dobson, Holman and Cutting (93) obtained an apparent cure from the use of sulfanilamide, iodides, and roentgen rays in the treatment of three cases of actinomycosis.

Dobson and Cutting (92) treated 16 cases with sulfonamide or penicillin. In 7 cases, the disease was considered as cured and as arrested in another 7. In three cases, penicillin alone was effective; in

three, penicillin and sulfadiazine were required; and six cases were cured by sulfonamide drugs. In two cases in which sulfonamide medication was given, the disease ended fatally. The conclusion was reached that both penicillin and sulfonamides are highly effective drugs in the treatment of the anaerobic forms of actinomycosis. A detailed study of the effectiveness of penicillin on various actinomycetes, including the representatives of the different genera, has recently been made by Drake (95).

Patients suffering from infections due to the aerobic *N. asteroides* have benefited from treatment with the sulfonamide compounds and penicillin; this benefit was similar to the effect upon patients infected with the anaerobic *A. bovis*. Surgical drainage, iodides, and roentgen ray therapy are recommended as the indications arise (92). Two cases of pulmonary and chest wall infections with acid-fast *Nocardia* gave good response to rest treatment, surgery, vitamins, sulfonamides and iodides (30).

Numerous contributions have recently been made concerning the treatment of actinomycosis with penicillin, alone (248) or in combination with sulfadiazine (93, 119, 209, 345). However, in a case of nocardiosis which resembled pulmonary tuberculosis, only intensive therapy with sulfadiazine was recommended; penicillin and streptomycin failed (139).

Actinomycosis of the central nervous system responded clinically to treatment with sulfadiazine, penicillin and streptomycin (183). Holm (177) surveyed the penicillin-sensitivity of anaerobic actinomycetes. Their sensitivity was found to be similar to that of staphylococci. If whole colonies were used, however, in making the tests, some were found to be more resistant. The resistance of the typical "sulfur granules" in the pus to penicillin may be due to this phenomenon. The dosage and mode of administration of penicillin should therefore be controlled by the presence of such granules.

Various strains of *A. bovis,* including both human and bovine isolates, were found to be highly sensitive to penicillin, all being inhibited by a concentration of 0.5 unit per milliliter. They developed only slight resistance to penicillin upon continuous transfer in media containing this antibiotic. The strains were inhibited by 30 units per milliliter of streptomycin. All strains rapidly developed a high degree of resistance upon consecutive transfer in media containing streptomycin. Both resistance and reversion to original sensitivity occurred in a step-wise manner suggesting the possibility of genetic changes in the organism (39a).

Chapter XII

SUMMARY

Actinomycetes are among the most widely distributed groups of microorganisms. They are of universal occurrence and they play an active part in the cycle of life in nature.

One of the early students of the group, BEIJERINCK, recognized that they are omnivorous organisms and that they are capable of living both in a nutrient-rich and in a very poor environment. Water and air were said to supply nutrients for the modest needs of these organisms.

Actinomycetes are able to utilize both inorganic and organic forms of nitrogen. The extent of their growth upon artificial media is governed by the available energy, the supply of oxygen, available nitrogen, and certain other nutrient elements. One of the greatest contributions to a better knowledge of these organisms was their cultivation on synthetic media, upon which they form characteristic morphological structures and upon which they develop a variety of specific biochemical characteristics.

Actinomycetes are capable of breaking down proteins to amino acids and to ammonia; frequently, active proteolytic enzymes are produced. They are able to utilize a large variety of organic compounds for nutritive purposes and grow under various favorable and adverse conditions. Many actinomycetes are strongly diastatic, and many are capable of attacking various hemicelluloses. Some are able to utilize cellulose, some attack lignins, paraffins, fats, and rubber-like materials.

Many actinomycetes are able to reduce nitrate to nitrite, but not to ammonia or atmospheric nitrogen. BEIJERINCK believed, however, that under certain conditions this reduction may lead to losses of nitrogen through the interaction of nitrites with ammonium compounds.

Actinomycetes are unable to fix atmospheric nitrogen, certain reports to the contrary notwithstanding (32). These reports were based upon the observation that many actinomyces colonies develop on media to which no fixed form of nitrogen has been added. The limited growth produced by such colonies can easily have obtained their nitrogen from various impurities in the medium or in the atmosphere. Actinomycetes do not nitrify ammonium salts, although detection of small amounts of nitrites has been reported (308) in media containing ammonia; this may have been due to the sensitivity of nitrite reagents.

Certain actinomycetes can develop at temperatures as high as 60°

to 65°C., especially in composts, whereas others, such as those found in abundance in muds and in lake and river bottoms, thrive at rather low temperatures. Under unfavorable conditions, actinomycetes grow only slowly and poorly; this has often raised the question concerning their active participation in a given process. Most of them are sensitive to an acid reaction (*p*H 6.0) and are favored by an alkaline reaction (*p*H 7.0 to 7.5) of the medium.

Actinomycetes produce a variety of pigments. The black pigment formed on protein media may function as an oxidizing agent, and on this basis, the suggestion has been made that actinomycetes play an important role in the formation of humus in the soil. They occur in soil at considerable depths, where they may exceed in numbers the other groups of microorganisms. These facts have led to the suggestion that actinomycetes play an important role in soil processes.

Of the four genera now recognized among the actinomycetes—*Actinomyces, Nocardia, Streptomyces,* and *Micromonospora*—the animal pathogens are found largely in the first, the anaerobic genus, and to some extent in the second. The plant pathogens are found in the third. The water forms and the high-temperature compost forms are found largely in the fourth. The last three genera occur in great abundance in soils, where they make up nearly 25 per cent of the total population of microorganisms developing on the ordinary agar plate. They occur in the dust and on the surface of grasses and foodstuffs. Their relative abundance in close proximity to the roots of plants is due not so much to their particular preference for living roots as to the fact that they find nourishment in the dead residues and excreta of the roots.

With the rapid progress, within recent years, of our knowledge of antibiotic substances, and with the recognition that actinomycetes may play an important role in the production of such agents, new interest was aroused in the nutrition of these organisms. The introduction of the submerged culture method for their cultivation has made possible not only their rapid and abundant growth but also the study of many physiological reactions not previously recognized.

In order to obtain abundant growth, sufficient energy material must be applied by proteins, carbohydrates, or organic acids; proper sources of nitrogen, either organic or inorganic; and certain minerals, notably potassium, magnesium, phosphorus, sulfur, and iron, are also necessary. Certain forms are capable of producing vitamin-like substances favoring the growth of other microorganisms. Many are able to produce antibiotic substances injurious to the growth of other organisms.

Under comparable conditions of nutrition, actinomycetes may produce as much growth and decompose as much of the substrate as do some of the common fungi and bacteria. Carbohydrates, such as glucose, favor the growth of the organism and the utilization of proteins and amino acids. The effect, however, is different from that upon fungi, since the latter prefer the carbohydrates to the nitrogen com-

pounds as sources of energy, whereas the actinomycetes prefer to utilize for this purpose the organic nitrogenous compounds. The glucose may thus serve as a buffer, since the acid produced from it tends to neutralize the excessive amounts of ammonia which are produced by actinomycetes and which would soon bring growth to a standstill because of a rapid change in reaction of the medium to highly alkaline, *p*H 8.6-9.0.

The metabolic changes produced in the medium by actinomycetes are greatly influenced by the nitrogen source. In general, however, actinomycetes are similar to bacteria and to fungi in their nutrition, in their energy utilization, in the transformation of nitrogenous compounds, in the liberation of ammonia, and in cell synthesis.

Actinomycetes thus form a major group of microorganisms, and comparable to the other two major groups, the bacteria and the fungi, their activities can be summarized under the following five headings:

Role in natural processes.
Causative agents of disease.
Agents of spoilage and deterioration.
Utilization for production of enzymes and vitamins.
Production of antibiotics.

Role of Actinomycetes in Natural Processes:—The general occurrence of actinomycetes in all soils and their omnivorous nature suggest their probable importance in soil processes. The facts that they make up as many as 15 to 40 per cent of all colonies developing on the plate, that they occur in the soil at great depth, and that they are favored by arid soil conditions and by an alkaline reaction suggest that, under certain conditions, actinomycetes are concerned in a number of important processes.

The following soil reactions may be due, to a considerable extent, to the activities of actinomycetes: *1.* Decomposition of complex plant and animal residues in soils and in composts. 2. Liberation of ammonia from complex proteins. 3. Humification processes accompanied by the formation of black coloring substances, the decomposition of humus compounds, and the synthesis of cell material, which further contributes to the formation of soil organic matter or soil humus. *4.* Reduction of nitrate to nitrite, but not to atmospheric nitrogen. 5. Favorable effects upon plant growth. For example, when a soil was enriched with actinomycetes, plant roots were longer. This effect was explained by the greater decomposition of organic soil constituents. The effect was greatest on legume bacteria, which suggested possible assistance to legume bacteria in infecting the plants and causing greater nodule development (125).

Actinomycetes appear to be important geological agents, although their role in this respect has not been fully established. Nadson (311), who isolated several actinomycetes from lake muds, found that they

could reduce gray mud to black mud, a process accompanied by movement of calcium and iron to the upper mud layers. The precipitation of $CaCO_3$ was said to be favored by the production of ammonia, which changes the reaction of the medium to alkaline. Molisch (302) included an *Actinomyces* among the organisms contributing to the precipitation of $CaCO_3$. Sawjalow (386) also isolated from lake mud an actinomyces (*A. pelogenes*) which was believed to be capable of reducing sulfate to hydrogen sulfide.

Actinomycetes as Causative Agents of Disease:—Actinomycetes, unlike the bacteria and the viruses, are not responsible for any of the great plagues that affect mankind and his domesticated animals. Neither are they as universal agents of plant destruction as are many fungi. Still, they are capable of causing certain important deep-seated diseases that affect both the animal and plant kingdoms. The actinomycotic diseases of man and animals and the scab diseases of certain plants, notably potatoes and mangels, point to their great potential importance as disease-producing agents.

Among the animal diseases, those brought about by anaerobic organisms (actinomycosis) and those brought about by aerobic forms (nocardiosis) are frequently confused. The introduction of penicillin as a chemotherapeutic agent has served to reduce the danger from these infections, at least so far as man is concerned.

The problem of plant diseases may sometimes reach alarming proportions in connection with the highly important economic crop the Irish potato. Scabbiness is favored by dry soil conditions, by an alkaline reaction, and by a high humus content of the soil. On the other hand, the use of organic fertilizers and green manures, especially under humid conditions, serves to control this infection. In addition to potato scab and mangel scab, a few other plant diseases, such as those of the sweet potato, are caused by actinomycetes, but these are only minor in nature.

Actinomycetes as Agents of Spoilage and Deterioration:—Actinomycetes may play a far more important role as agents of spoilage than is commonly appreciated. This includes two phenomena: *1.* Deterioration of certain foodstuffs, which is largely caused by the imparting of characteristic earthy and pungent flavors and odors to milk, cacao, potable waters, and fish. In the case of the latter it is not the direct infection of the fish but the tainting of their flesh due to the absorption of the odoriferous substance from the water. 2. Staining and actual destruction of certain fabrics, notably, woolens, cotton goods, and paper. Actinomycetes cannot compare with the fungi as agents of destruction of textiles under humid and high temperature conditions. But even as slower-growing organisms, they can produce on cloth, either woolen or cotton, and on paper, especially in books, stains which reduce considerably the value of the material.

Utilization of Actinomycetes for the Production of Enzymes and Vitamins:—Comparatively little use has been made so far of actinomycetes for production of chemical compounds that find application in industry or in nutrition. Only one attempt has been made to utilize the diastatic enzyme of an actinomyces; this has been produced under the name "superbiolase," because of its ability to withstand higher temperatures than the corresponding enzymes of barley and of certain microorganisms. Of much greater importance is the recent finding (359a) that certain strains of *S. griseus* (grisein-producing) are capable of producing vitamin B_{12}. The red crystalline material isolated from these cultures had all the properties of the compound isolated from liver. These crystals possessed optimal "animal protein factor" activity for the chick at a level of 30 μg/kg of diet, similar to that found for vitamin B_{12}.

Production of Antibiotics:—Among the various groups of microorganisms that have the capacity to produce antibiotic substances, or agents which have the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms, the actinomycetes occupy a prominent place. Within the last 7 or 8 years, nearly 30 antibiotics have been isolated. They vary greatly in their antibacterial properties or in their antibiotic spectrum, in their chemical nature, in their toxicity to animals, and in their chemotherapeutic potentialities. Some, like actinomycin, are highly toxic; others, like streptomycin, possess only a very limited toxicity. Some are produced by more than one organism; and some organisms produce more than one antibiotic.

Of the various antibiotics produced by actinomycetes, streptomycin occupies a leading place. First announced in January 1944, it was used clinically within less than 2 years. Among its most striking properties are its action against gram-negative bacteria and the bacteria causing tuberculosis. Thus, a chemotherapeutic agent that has marked effects against the "white plague" of man has been discovered. What appeared only a few years ago to be one of the greatest scourges affecting millions of human beings has been subjected to control by the product of an actinomyces. Within 5 years after its announcement, the production of this antibiotic has risen to nearly 8 million grams per month.

Some of the newer antibiotics, notably aureomycin and chloromycetin, have also attained remarkable production records.

The possibility of discovering other antibiotics that would supplement streptomycin or take a place by its side as an important therapeutic agent appear very promising. Although some agents, like streptothricin, appear to be too toxic to offer great immediate promise, others, like grisein, are highly active and possess only very limited toxicity. These, therefore, appear promising.

Thus, the actinomycetes have contributed important tools for combating human and animal infections. The end of these possibilities is

not yet in sight. Of what significance these reactions are to soil processes still remains to be determined.

The actinomycetes can take their place among the major groups of microorganisms affecting the economy of man in numerous ways. Their importance in the cycle of life in nature and in the control by man of natural processes can hardly be exaggerated.

APPENDIX

MEDIA USED FOR THE STUDY OF ACTINOMYCETES

1. *Czapek's agar:*

$NaNO_3$	2 gm
K_2HPO_4	1 gm
$MgSO_4.7H_2O$	0.5 gm
KCl	0.5 gm
$FeSO_4$	0.01 gm
Sucrose	30 gm
Agar	15 gm
Distilled water	1000 ml
*p*H 6.6	

2. *Glucose-asparagine agar:*

Glucose	10 gm
Asparagine	0.5 gm
K_2HPO_4	0.5 gm
Agar	15 gm
Distilled water	1000 ml
*p*H 6.8	

3. *Glycerol agar:*

Glycerol	10 gm
Sodium asparaginate	1.0 gm
K_2HPO_4	1.0 gm
Agar	15 gm
Tap water	1000 ml
*p*H adjusted to 7.0	

4. *Tyrosin agar:*

Glucose	10 gm
Tyrosin	1 gm
$(NH_4)_2SO_4$	0.5 gm
K_2HPO_4	0.5 gm
Agar	15 gm
Distilled water	1000 ml
Reaction made neutral with NaOH	

5. *Meat-peptone agar:*

Peptone	5 gm
Meat extract	5 gm
NaCl	5 gm
Agar	15-20 gm
Tap water	1000 ml
*p*H 7.2-7.4	

6. *Glucose-peptone agar A:*

Peptone	5 gm
Glucose	20 gm
NaCl	5 gm
Agar	15 gm
Distilled water	1000 ml
*p*H 7.2	

7. *Glucose-peptone agar B:*

Peptone	5 gm
Glucose	10 gm
KH_2PO_4	1 gm
$MgSO_4.7H_2O$	5 gm
Agar	15 gm
Distilled water	1000 ml

8. *Meat-peptone gelatin:*

Peptone	5 gm
Meat extract	5 gm
Gelatin	100 to 200 gm
Tap water	1000 ml
Adjust to *p*H 7.4	
Sterilize 30 minutes at 110°C.	

9. *Peptone gelatin:*

Peptone	5 gm
Glucose	20 gm
Gelatin	100 to 200 gm
Tap water	1000 ml
Adjust to *p*H 7.2	
Sterilize 30 minutes at 110°C.	

10. *Starch agar A:*

Potato starch (corn starch or soluble starch)	10 gm
K_2HPO_4	0.3 gm
$MgCO_3$	1.0 gm
NaCl	0.5 gm
$NaNO_3$	1.0 gm
Agar	15 gm
Distilled water	1000 ml
Neutralize	
Sterilize 30 minutes at 110°C.	

11. *Starch agar B:*

Soluble starch	2 gm
K_2HPO_4	0.5 gm
$MgSO_4.7H_2O$	0.2 gm
$CaCl_2$	0.05 gm
$NaNO_3$	0.05 gm
Asparagine	0.05 gm
$Fe_2(SO_4)$	Trace
Washed agar	20 gm
Distilled water	1000 ml
*p*H 7.4	

12. *Egg albumen agar:*

Glucose	10 gm
K_2HPO_4	0.5 gm
$MgSO_4.7H_2O$	0.2 gm
$Fe_2(SO_4)_3$	Trace
Egg albumen	0.15 gm
Agar	15 gm
Distilled water	1000 ml

Egg albumen is first dissolved in water and made neutral to phenolphthalein with N/10 NaOH.

13. *Potato-nutrient agar:*

Peeled potatoes	500 gm
Peptone	10 gm
Meat extract	10 gm
NaCl	5 gm
Agar	15 gm
Tap water	1000 ml

*p*H 7.0

The potatoes are cut into small cubes to which 350 ml of water is added and the whole steamed for three-quarters of an hour. The extract is strained through fine muslin without squeezing the pulp. The other nutrients are dissolved in 350 ml of water which is then added to the potato extract, and the whole steamed for three-quarters of an hour. The mixture is then made up to bulk, standardized and filtered, after which the agar is added.

14. *Potato-glucose agar:*

Peeled potatoes	300 gm
Glucose	5 gm
Agar	20 gm
Tap water	1000 ml

*p*H 6.8

15. *Starch nitrate agar:*

Formula same as in medium 19, with addition of 1.5 per cent agar.

16. *Glucose broth:*

Glucose	10 gm
Peptone	5 gm
Meat extract	5 gm
NaCl	5 gm
Distilled water	1000 ml

*p*H 7.1

Frequently tap water is used, as in media for the production of streptomycin.

17. *Nutrient broth:*

As above, but free from glucose.

18. *Sucrose solution:*

Same as for No. 1, free from agar.

19. *Starch solution:*

Soluble starch	20 gm
K_2HPO_4	1 gm
$MgSO_4.7H_2O$	0.5 gm
KCl	0.5 gm
$NaNO_3$	2 gm
$CaCO_3$	2 gm
Distilled water	1000 ml

Starch is made into a paste and boiling water added. Mixture steamed for an hour to give a clear solution.

20. *Yeast extract medium A:*

Autolyzed yeast extract	2.5 gm
Glucose	5 gm
Distilled water	1000 ml

*p*H 6.0 to 7.0 (383)

21. *Yeast extract medium B:*

Yeast extract	10 gm
Glucose	10 gm
NaCl	5 gm
$MgSO_4.7H_2O$	0.25 gm
$FeSO_4.7H_2O$	0.01 gm
Distilled water	1000 ml

22. *Yeast extract agar:*

Yeast extract	10 gm
Glucose, technical	10 gm
Agar	15 gm
Tap water	1000 ml

*p*H 6.8

23. *Emerson's medium:*

Beef extract	4.0 gm
Peptone	4.0 gm
NaCl	2.5 gm
Yeast extract	1.0 gm
Glucose	10.0 gm
Distilled water	1000 ml

24. *Corn steep medium:*

Peptone	5.0 gm
Corn steep	15.0 gm
NaCl	5.0 gm
Glucose	10.0 gm
Distilled water	1000 ml

25. *Soybean medium:*

Soybean meal	10.0 gm
Commercial glucose	10.0 gm
NaCl	5.0 gm
Curbay B.G.	0.5 gm
$CaCO_3$	1.0 gm
Distilled water	1000 ml

26. *Synthetic lactate medium:*

Glucose	7.4 gm
KH_2PO_4	2.38 gm
$K_2HPO_4.3H_2O$	5.65 gm
NH_4 lactate	5.4 gm
$MgSO_4.7H_2O$	0.98 gm
$ZnSO_4.7H_2O$	11.5 mg
$FeSO_4.7H_2O$	11.1 mg
$CuSO_4$	6.4 mg
$MnCl_2.4H_2O$	7.9 mg
Distilled water	1000 ml
*p*H 6.95	

27. *Potato plugs.*

28. *Carrot plugs.*

29. *Brom-cresol milk:*

Prepared according to formula of Clark and Lubs. Medium sterilized by steaming for 20 minutes on three successive days.

30. *Dorset's egg or glycerol egg medium.*

31. *Brain-heart infusion with 2 per cent agar* (368).

A number of other special media are used, either for the growth of organisms or for the production of special substances, notably antibiotics. In several media, the peptone is replaced by casein hydrolysate and the meat extract by various digests of plant residues, such as soy bean meal or by waste products of manufacturing industries, such as corn steep liquor or by fermentation residues, such as distillery slops.

Actinomyces bovis,

ein neuer Schimmel in den Geweben des Rindes.

Von

Dr. **C. O. Harz.**

1. Geschichtliches.

Seit nahezu 10 Jahren besitzen wir Kenntniss von der Existenz mikroskopisch kleiner nicht zu den Schizophyten Cohn's gehöriger Parasiten, welche in das Innere der Gewebe des lebenden Thierkörpers einzudringen, darin sich zu vermehren und gefährliche Erkrankungen sowie den Tod ihres Wirthes zu bedingen vermögen.

Es sind dies theils evident pflanzliche, theils dem sog. Protistenreiche angehörige Organismen von charakteristischer Form; vermöge ihres Umfanges sofort erkennbar, ohne Schwierigkeit von verwandten Gebilden zu unterscheiden und theilweise leicht in allen Entwickelungsstadien zu verfolgen, sind sie als Krankheitsursache mit Sicherheit meist leicht zu erkennen.

Ursache und Wirkung begleiten sich hier stets in so auffallender Weise, dass es unter den zahlreichen Forschern, die sie in Wirklichkeit beobachtet haben, auch nicht Einem einfiel, an ihrer pathogenen Thätigkeit zu zweifeln.

Ueber die Ernährungsweise dieser Parasiten ist wenig bekannt. Da sie von den Bestandtheilen des von ihnen bewohnten thierischen Körpers leben, schädigen sie den letzteren nicht nur durch fortgesetzten Verbrauch seiner Substanz, sondern auch durch continuirliche Verunreinigung (Vergiftung) in Folge der aus den Zersetzungen organischen Körper gebildeten, sowie der eigenen Ausscheidungsprodukte.

Es treten endlich Störungen der verschiedensten Art auf in Folge massenhafter Anhäufung der sich vermehrenden Parasiten, unter Umständen tritt Erstickungstod in Folge Sauerstoffentzuges durch denselben ein.

Den freien Sauerstoffbedarf scheinen sie aus dem im Thierkörper existirenden Vorrathe mit Leichtigkeit decken zu können,

Fig. 39.—First use of the generic name *Actinomyces* (C. O. Harz: *Actinomyces bovis*, ein neuer Schimmel in den Geweben des Rindes *in* Deut. Zeitsch. Thiermed. 5:125-140, 1877/78).

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